Improving Prediction Strategies in Rheumatoid Arthritis:

Additional Predictive Ability of Synovial Pathotype over

Clinical, Laboratory and Imaging Findings

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory disease of autoimmune origin affecting approximately 1% of adult population worldwide. The clinical course of RA is highly variable, ranging from self-limiting to severe disease, with considerable individual and socio-economic implications.

It is now well acknowledged that early diagnosis and treatment equates to better long-term outcomes.

However, despite major therapeutic advances in recent decades, the management of RA remains challenging as a significant proportion of patients presents with active disease despite maximization of therapy. It is also difficult to predict which patients will respond adequately to various treatment regimens. The identification of biomarkers of clinical outcome capable of stratifying patients into accurate prognostic categories and guide pharmacological intervention is therefore urgently needed.

Notably, along with clinical variability, RA is characterised by high biological heterogeneity at the tissue level. The cellular infiltrate of the RA synovium can be distinguished into at least three main patterns according to the degree and organisation of the immune cells: the 'Lymphoid' pattern characterised by predominant B and T lymphocytes which tend to cluster in discrete aggregates resembling ectopic lymphoid structures; the 'Myeloid' pattern characterised by absence of lymphocytic aggregates but significant expression of sublining macrophages; the 'Pauci-immune' pattern, that hardly shows any infiltrating immune cells.

The hypothesis of this thesis was to determine whether these distinct synovial pathotypes may define specific disease subsets and predict response to therapy in patients with RA.

Specifically, this work aims at:

1. evaluating whether the synovial pathotype associates with the presence of specific clinical, serological, radiological and ultrasonographic findings in an early RA cohort (< 1 year onset);

2. exploring the potential role of the synovial pathotype as a predictor of response to conventional synthetic disease-modifying antirheumatic drugs (csDMARD) after 6 months in an early RA cohort;

3. exploring the potential role of the synovial pathotype as a predictor of response to anti-TNFα treatment after 3 months in a csDMARD-failure established RA cohort.

DECLARATION

I declare that the following thesis has been composed by myself, that it embodies the results of my own special work, and that it does not include work forming part of a thesis presented successfully for a degree at this or any other university.

Maria Di Cicco

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LIST OF ABBREVIATIONS

ACPA	anti-citrullinated protein antibodies
ACR	American College of Rheumatology
ADAMTS	A-disintegrin and metalloproteinase with thrombospondin-1-like domains
ADCC	antibody-dependent cell-mediated cytotoxicity
AID	Activation-Induced Cytidine Deaminase
AMPD1	adenosine monophosphate deaminase1
Anti-CCP1	anti-cyclic citrullinated proteins antibodies 1
Anti-CCP2	anti-cyclic citrullinated proteins antibodies 2
AP	antero-posterior
APC	antigen presenting cells
APF	antibody perinuclear factor
APRIL	proliferation-inducing ligand
ARAMIS	Arthritis Rheumatism and Aging Medical Information System
ASPIRE	'Active-Controlled Study of Patients Receiving Infliximab for the Treatment of Rheumatoid Arthritis of Early Onset'
ATTRACT	'Infliximab Versus Placebo in Rheumatoid Arthritis Patients Receiving Concomitant Methotrexate: a Randomised Phase III Trial'
BAFF	B cell activating factor
bDMARD	biologic Disease-Modifying Antirheumatic Drugs
BeSt	'Behandel Strategieën'
BLyS	B lymphocyte stimulator
CAMERA	'Computer Assisted Management for Early Rheumatoid Arthritis'
CC	CC-chemokine
CCL19	CC-chemokine ligand 19
CCL21	CC-chemokine ligand 21
CDC	complement-dependent cytotoxicity
CD40L	CD40 ligand
CI	confidence intervals
CIA	collagen induced arthritis

CLIP-Cert	'Clinical responsiveness to anti-TNFα therapy and modulation of synovial lymphoid structures and B cell function in RA- An exploratory open label prospective study in RA'
COBRA	'Combinatie therapie Bij Reumatoide Artritis'
COMET	'Comparison of Methotrexate Monotherapy with a Combination of Methotrexate and Etanercept in Active, Early, Moderate to Severe Rheumatoid Arthritis'
COX-2	cyclo-oxygenase 2
CRP	C-reactive protein
csDMARD	conventional synthetic Disease-Modifying Antirheumatic Drugs
CSF	granulocyte–macrophage colony-stimulating factor
CTLA4	cytotoxic T lymphocyte associated antigen 4
CV	cardiovascular
CXC	CXC-chemokine
CXCL13	CXC-chemokine ligand 13
CZP	Certolizumab pegol
DAMPs	damage associated molecular patterns
DAS28	28 joint count-Disease Activity Score
DC	dendritic cells
DIA	digital image analysis
DIPs	distal interphalangeal joints
Dkk-1	dickkopf-1
DMARD	Disease-Modifying Antirheumatic Drugs
DNA	deoxyribonucleic acid
EAC	early arthritis clinic
EAM	extra-articular manifestations
EBV	Epstein-Barr virus
ELN	ectopic lymphoid neogenesis
ELS	ectopic lymphoid-like structures
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
Fab	fragment antigen-binding

Fc	crystallized fragment
FcR-γ	crystallized fragment receptor γ
FcεRI	high-affinity IgE receptor
FDC	follicular dendritic cells
FLS	fibroblast-like synoviocytes
FOXP3	forkhead box P3
FRAX	Fracture Risk Assessment Tool
G0,1,2, 3	grade 0,1,2,3
GATA3	GATA binding protein 3
GC	germinal centres
GI	gastrointestinal
GM-CSF	granulocyte monocyte-colony stimulating factor
GM-FGF	fibroblast growth factor
H&E	haematoxylin and eosin
HA	hyaluronic acid
HAQ	Health Assessment Questionnaire
HBV	hepatitis B
HCQ	Hydroxychloroquine
HCV	hepatitis C
HEV	high endothelial venules
HIFs	hypoxia-inducible factors
HIV	Human Immunodeficiency Virus
HLA	human leukocyte antigen
HSPs	heat shock proteins
ICC	intra-class correlation coefficients
IFNα	interferon α
IFNβ	interferon β
IFNγ	interferon γ
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M

IGRA	Interferon-Gamma Release Assay
IL-1	interleukine-1
IL-1Ra	interleukine-1 receptor antagonist
IL-2	interleukine-2
IL-4	interleukine-4
IL-5	interleukine-5
IL-6	interleukine-6
IL-6R	interleukine-6 receptor
IL-7	interleukine-7
IL-10	interleukine-10
IL-17	interleukine-17
IL-18	interleukine-18
IL-22	interleukine-22
IL-23	interleukine-23
IL-32	interleukine-32
IL-33	interleukine-33
IQR	interquartile range
JE	joint erosions score
JSN	joint space narrowing
kDa	kiloDalton
LEF	Leflunomide
LTα	lymphotoxin α
LTβ	lymphotoxin β
M-CSF	macrophage colony stimulating factor
MALT	mucosa associated lymphoid tissue
MAP	mitogen-activated proteins
MC	manual cell counting
MCPs	metacarpophalangeal joints
MHC	major histocompatibility complex
mi-RNA	micro-ribonucleic acid
MICE	multivariate imputation by chained equations
MIF	macrophage migration inhibitory factor

MMP-1	matrix metalloproteinase-1 (MMP-1)
MMPs	matrix metalloproteinases
MRC	Medical Research Council
MRI	magnetic resonance imaging
MTHFR	methylenetetrahydrofolate reductase
MTPs	metatarsophalangeal joints
MTX	Methotrexate
NF-kB	nuclear factor kB
NHS	National Health System
NICE	National Institute for Health and Care Excellence
NK	natural killer cells
NOR-DMARD	'Norwegian-disease modifying antirheumatic drugs'
NPV	negative predictive values
NRL	nucleotide-binding oligomerization domain-like receptor
NSAIDs	non-steroidal anti-inflammatory drugs
OMERACT	Outcome Measures in Rheumatology
OR	odds ratio
PAD	peptidyl arginine deiminase
PADI4	peptidyl arginine deiminase type 4
PAMPs	pathogen associated molecular patterns
PAR2	protease-activator receptor 2
PDGF	platelet-derived growth factor
PDUS	power doppler ultrasound
PEAC	'Pathobiology of Early Arthritis Cohort'
PEG	polyethylene glycol
PI	Pauci-immune
PIPs	proximal interphalangeal
PNAd	peripheral node addressins
PPAD	P. gingivalis peptidyl-arginine deiminase
PPRs	pattern recognition receptors
PPV	positive predictive values
PRED	Prednisolone

PREMIER	'Efficacy and Safety of Adalimumab and Methotrexate versus Methotrexate Monotherapy in Subjects with Early Rheumatoid Arthritis'
PROMPT	'PRObable rheumatoid arthritis: Methotrexate versus Placebo Treatment'
PsA	psoriatic arthritis
PTPN22	protein tyrosine phosphatase N22
PUK	peripheral ulcerative keratitis
QMUL	Queen Mary University of London
RA	rheumatoid arthritis
RANK	receptor activator of nuclear factor κ-B
RANKL	receptor activator of nuclear factor κ-B ligand
RCTs	randomised controlled trials
RF	rheumatoid factor
RNA	ribonucleic acid
RNPs	ribonucleoproteins
ROI	region of interest
RORC2	retinoic acid receptor-related orphan receptor C2
RR	relative risk
SCID	Severe Combined Immunodeficiency
SD	standard deviation
SE	shared epitope
ShSS	van der Heijde modified Sharp score
SJ	swollen joints
SQ	semi-quantitative
SSZ	Sulfasalazine
STAT4	signal transducer and activator of transcription 4
STRAP	'Stratification of biologic Therapies for Rheumatoid Arthritis by Pathobiology'
STUS	synovial thickening ultrasound
T2T	treat to target
TaSER	'Targeting ultrasound remission in rheumatoid arthritis'

TB	tuberculosis
TBX21	T-box transcription factor 21
TCR	T cell receptor
TEMPO	'Trial of Etanercept and Methotrexate with Radiographic Patient Outcome'
TGFβ	transforming grow factor β
Th	T helper cell
Th0	type 0 T helper cell
Th1	type 1 T helper cell
Th17	type 17 T helper cell
Th2	type 2 T helper cell
TICORA	'Tight Control of Rheumatoid Arthritis'
TJ	tender joints
TLRs	toll like receptors
TNF-RI	tumour necrosis factor-receptor I
TNF-RII	tumour necrosis factor-receptor II
TNFα	tumour necrosis factor α
TRAF5-C1	TNF receptor associated factor 5-C1
Treg	regulatory T cells
tsDMARD	targeted syntetic Disease-Modifying Antirheumatic Drugs
TUI	Targeted Ultrasound Initiative
TURA	'Targeted Ultrasound in Rheumatoid Arthritis'
UA	undifferentiated arthritis
UK	United Kingdom
US	ultrasound
VAS-GH	Visual Analogue Score for Global Health
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
XR	radiographs

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Chapter 1 : INTRODUCTION

1.1 DEFINITION OF RHEUMATOID ARTHRITS

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease of unknown origin, primarily affecting the synovial joints however characterised by a broad spectrum of extra-articular manifestations. The clinical course of RA is variable, ranging from mild to severe disease, which can potentially lead to joint damage, chronic disability and early mortality. This translates to considerable costs at the individual, societal and economic level. [1]

1.2 EPIDEMIOLOGY

RA affects approximately 1% of the adult population worldwide. [2]

The prevalence varies geographically and among population groups, suggesting a role for genetic background and environmental exposure. However, epidemiology studies on RA are affected by methodological issues, partially because the methods of case identification and case recording have changed over time. In 1987 the revised American College of Rheumatology (ACR) criteria replaced the previous existing criteria (1958 New York classification criteria). [3] More recently, the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2010 criteria have been introduced. [4] Also, the small number of studies for most areas of the world and the lack of incidence studies for developing countries must be taken into account.

A trend towards higher prevalence and incidence in northern Europe and North America compared to developing countries has been observed, to an extent that RA is virtually undetectable in rural areas of Africa and China. Interestingly, Africans who migrate from rural areas towards urbanised and industrialised

societies present with an increased risk compared to their counterparts who did not migrate, stressing the importance of environmental factors. The disease is also less common in southern compared to northern Europe. [5]

In certain human groups, where specific genes may have segregated due to geographical and social isolation, the prevalence may differ consistently. Among Native Americans and Alaskans, for example, rates of up to 5.3-6% have been reported. [3]

The prevalence of RA is estimated to be around 0.8% in the United Kingdom. [6]

The peak age of onset is the 5th to 6th decade, and the prevalence increases with age. The disease is more common in women, with a male to female ratio of 1:3, suggesting the importance of genetic and possibly a role for hormonal influence in disease pathogenesis. [3]

A general trend for a decrease in both incidence and prevalence of the disease over the past few decades has been registered worldwide. [5]

1.3 AETIOLOGY

Significant advances in understanding the pathogenesis of the disease have been made in recent years, leading to the discovery of new potential targets and effective therapies, and ultimately to a significant improvement in clinical outcome.

Unfortunately, despite such remarkable progresses, the aetiology of RA remains unknown. The current paradigm is that the disease is the result of a complex interplay between genetic and environmental factors, most of which have not been identified yet. [1]

1.3.1 Genetics

The disease is known to cluster in families. The individual risk of developing RA is between 2 and 4 fold higher when a first-degree relative is affected. [7]

Genes may have a substantial contribution to RA, and it has been estimated that up to 60% of risk is attributable to genetic factors. Twins studies show that concordance rates for RA are higher in monozygotic (15-30%) as opposed to dizygotic twins (5%) and higher in dizygotic twins than general population. [8]

Over 30 genetic regions have been identified in association with RA, however each single allele provides only modest contribution to the global genetic susceptibility. Taken together, currently known genetic risk factors for RA only explain ~16% of the susceptibility on an individual basis. Overall, if on one hand this data supports the genetic component of RA, on the other hand implies that genes alone are insufficient to explain the disease pathogenesis, suggesting a concomitant role for environmental factors.

Class II human leukocyte antigen (HLA) and non-HLA genes have been implicated in susceptibility to RA. A potential association between HLA alleles and RA was first reported in 1969. [9] Subsequent studies showed that the region HLA-DR may account for up to one third on the genetic risk in RA [10], although recent data suggested that this figure may represent an overestimation. Specifically, it has been observed an association with HLA-DRB1 alleles, which are located on the short arm of chromosome 6 (6p21.3). HLA-DRB1 alleles share a common motif -a sequence of amino acids in the third hypervariable region of the β chain of the HLA-DR, known as the shared epitope (SE). This sequence occurs within the antigen binding site of the major histocompatibility complex (MHC) molecule,

which influences the antigen presentation to T cells, suggesting a possible alteration in T-cell repertoire selection, antigen presentation and antigen affinity, ultimately resulting in a dysregulation of the innate immune system and antigen-driven T and B cell activation. [10]

HLA-DRB1 is associated with the development of certain autoantibodies specifically found in RA, anti-citrullinated protein antibodies (ACPA). [11] The contribution of HLA alleles to the genetic variance has been estimated at 40 % for ACPA seropositive and only 2 % for ACPA negative patients, suggesting that ACPA-positive and ACPA-negative RA maybe genetically distinct subsets. [12]

Some HLA-DR4 subtypes, such as Dw10 and Dw13, have been negatively associated with RA. [13]

Among the non-HLA associated genes, a well-known association is with PTPN22 (protein tyrosine phosphatase N22), a gene encoding for an intracellular enzyme which negatively regulates T cell activation. [14] Such association has been mostly observed in ACPA seropositive patients.[15]

Potential associations have been observed with other non-HLA genes involved in several immune-regulatory process, including: nuclear factor kB (NF-kB) dependent signalling[16], proteins regulating T cell activation such as cytotoxic T lymphocyte associated antigen 4 (CTLA4)[17], enzymes involved in protein citrullination like peptidyl deiminase type 4 (PADI4)[18], signal transducers such as signal transducer and activator of transcription 4 (STAT4)[19], proteins regulating T cell receptor and TNF receptor signalling such as TNF receptor associated factor 5-C1 (TRAF5-C1)[20], macrophage inhibitory factor (MIF)[21]. Nonetheless, the contribution of these associations seems to be relatively modest, as they have

emerged from limited studies only, with inconsistent results obtained across different populations.

1.3.2 Epigenetics

Epigenetics is emerging as a key pathogenic mechanism in several human diseases, including cancer and autoimmunity, with prospects for future targeted therapies.

Epigenetics defines stable although potentially reversible changes in gene expression which does not involve changes in the nucleotide sequence, but is rather the result of intra-cellular biochemical signals that can activate or silence genes. The transcription of deoxyribonucleic acid (DNA) is ultimately regulated by sophisticated extra-genetic mechanisms. This regulatory system is not fixed but extremely dynamic and influenced by environmental factors. Epigenetic mechanisms include DNA methylation, post-translational histone modification and micro-ribonucleic acid (mi-RNA). [22] These pathways are suspected of being involved in typical immune alterations observed in RA, such as the generation of auto-reactive T and/or B cell clones or the stromal cells activation within the inflamed synovium. [23] As an example, it has been observed that the expression of miRNA-203 is up-regulated in RA synovial fibroblasts, and this overexpression is associated with the secretion of mediators of local inflammation and damage such as interleukine-6 (IL-6) and matrix metalloproteinase-1 (MMP-1). [24] This is an intriguing and expanding field of research, as epigenetics may represent the key link between genetic susceptibility and exposure to environmental factors underlying the pathogenesis of RA.

1.3.3 Environmental factors

1.3.3.1 Infectious agents

A number of infectious agents have been suggested as putative pathogenic triggers for RA, including Epstein-Barr virus (EBV)[25], Human retrovirus[26], Parvovirus B19[27], Mycoplasma[28] and many others. Several immunological mechanisms have been hypothesised, and more than one could be involved simultaneously: molecular mimicry (structural similarities between microbial and self-antigens causing immune cross-reactivity), bystander activation, polyspecific B cell activation, accumulation of infectious agent-specific CD8+ T-cells in sites of inflammation, and epigenetic dysregulation. [29]

RA is known to be associated with an increased EBV viral load in the synovial fluid, peripheral blood and synovial tissue, as well as with high titres of anti EBV-antibodies and cross-reactive circulating antibodies to viral antigens. [30,31] Recent data also suggests that persistency of auto-reactive plasma cells to EBV in the synovial lymphoid structures of RA patients is associated with local differentiation of ACPA-reactive B cells, raising the hypothesis that EBV infection may play a direct role in the production of these autoantibodies.[32]

A role in the pathogenesis of RA has also been recently postulated for Porphyromonas gingivalis, a pathogen responsible for chronic periodontitis. RA and periodontitis are known to be associated at an epidemiological level, as patients with chronic periodontitis have two-fold risk of developing RA and viceversa. [33] Interestingly, this organism produces a specific bacterial enzyme, P. gingivalis peptidyl-arginine deaminase (PPAD), which is capable of promoting peptide citrullination [34].

However, to date the specific contribution of a single infectious agent in the pathogenesis of the disease remains elusive.

1.3.3.2 Smoking

The relationship between smoking and autoimmunity is widely known. Smoking has the capacity to induce epigenetic changes through the release of free radicals and other toxins, causing dysregulation of the immune system at various levels. Tobacco smoke can has also promote macrophage and dendritic cell activity and release of several pro-inflammatory cytokines.[35]

Specifically, smoking is a well established environmental risk factor for RA, as emerged from several epidemiological studies and case-control cohorts. [36-38] This risk appears to be dose-dependent and can persist up to 20 years after smoking cessation.[38]

A strong association between cigarette smoking and RA is observed within the context of HLA-DRB1 SE and presence of ACPA autoantibodies. [39] It has been estimated that the combination of the SE and smoking habit increases the relative risk of developing RA by 21 fold in ACPA seropositive patients. [40] To explain this association, a comprehensive immunopathological model has been proposed [1]: smoking may induce post-translational modifications of proteins expressed in the lungs via an up-regulation of certain enzymes such as peptidyl-arginine deiminase (PAD) expressed by macrophages, resulting in an accumulation of citrullinated peptides. In genetically susceptible people (e.g. individuals carrying the HLA–DR SE alleles, to which these post-translational modified epitopes can bind) this may lead to the persistent activation of the adaptive immune response and subsequent initiation of a specific anti-citrulline humoral response. For reasons which are not

entirely understood, and possibly after the intervention of a "second hit", the immune response can localise to other organs, particularly the synovium, perpetuating autoimmunity and inflammation in situ that may ultimately result into the development of chronic arthritis.[40]

Several studies have reported a familial association in the expression of ACPA antibodies, although whether such expression is related to shared genetic and/or shared environmental factors has not yet been addressed. [41-44] A recent analysis from the National Swedish Twin Register reported that environment, lifestyle and stochastic factors including smoking may be more relevant than genetics in inducing the development of ACPAs, however genetic factors, and especially SE, may have an important role in determining which ACPA-positive individuals will subsequently develop RA. [45]

It has been postulated that smoking status can also influence the clinical course of RA: several studies report that smokers tend to have a worse outcome, with greater radiological progression and loss of function [46,47], as well as a poorer response to therapy.[48] Whether such an association is direct or indirect -namely mediated by the contribution of smoking in development of ACPA antibodies which mark a more aggressive disease course- is unknown.

1.3.3.3 Hormonal and reproductive factors

Sex hormones, especially estrogens, have been proposed as having a role in susceptibility to RA. [49] This association is supported by several findings.

First, the previously mentioned 3 times higher prevalence in women compared to men, a ratio which tends to decrease after menopause.[3]

Second, pregnancy has a significant impact on the incidence of RA and its clinical course. Pregnancy is associated with a reduction in disease incidence of about 70% and overall with beneficial effects on disease activity and achievement of clinical remission. [50] This could be directly related to hormonal changes but also to maternal-fetal disparity in HLA class II alloantigens. [51] Conversely, an increased incidence of the disease and high rates of flares has been reported in the immediate post-partum and during the breastfeeding period, which has been partially attributed to the pro-inflammatory effects of prolactin. [52]

There is no strong evidence supporting the influence of exogenous hormones such us oral contraceptives or hormone replacement therapy [53], although a modest protective effect for oral contraceptives has been observed in small studies. [54]

1.4 IMMUNOPATHOLOGY

Immunopathology of RA is characterised by a complex interplay between adaptive and innate immune system, along with responses mediated by synovial resident cells. Indeed, the demarcation between these immunological compartments is a simplistic and artificial schematisation as in real life they crosstalk and integrate to form an inextricable network.

The currently accepted immunopathological model is that, in genetically susceptible individuals, environmental factors can induce citrullination of proteins and consequently cause a breach of peripheral immune tolerance to self-antigens, leading to inflammation and autoimmunity. Thereafter, the immune response can localise and perpetuate within the synovium, for reasons that are not fully

understood. Local micro-vascular, micro-environmental and biomechanical factors may contribute to facilitate this immune response within the joint tissue.[1] (Figure 1.1)

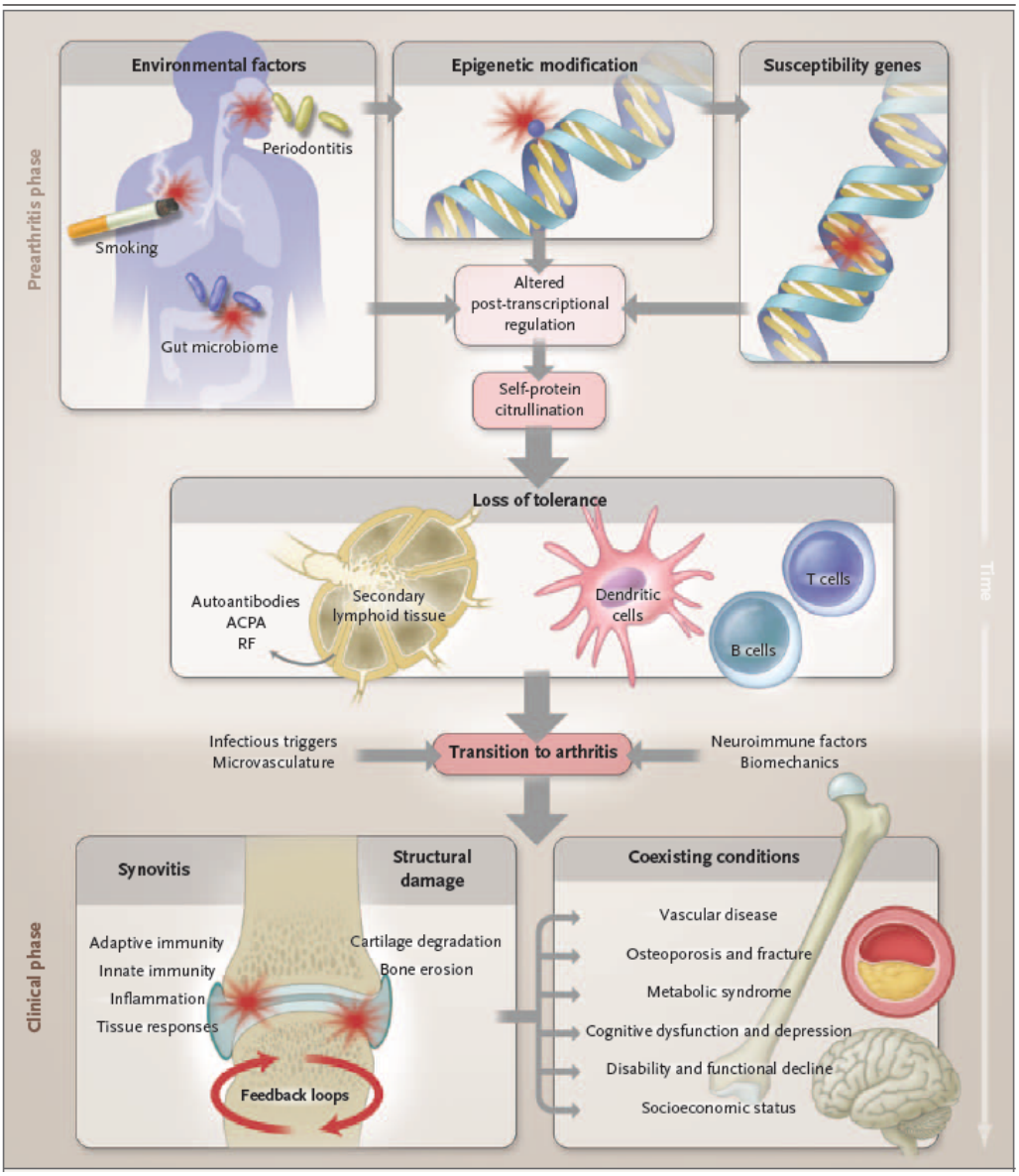


Figure 1.1 : Multistep progression to the development of rheumatoid arthritis
Environment–gene interactions promote loss of tolerance to self-proteins that contain a citrulline residue, which is generated by post-translational modifications. This anti-citrulline response can be detected in T-cell and B-cell compartments and is probably initiated in secondary lymphoid tissues or bone marrow. Thereafter, localisation of the inflammatory response occurs in the joint by poorly understood mechanisms that probably involve micro-vascular, neurological, biomechanical, or other tissue-specific pathways. Synovitis is initiated and perpetuated by positive feedback loops and in turn promotes systemic inflammation and potential extra-articular manifestations.
Abbreviations: ACPA = anti–citrullinated protein antibody; RF= rheumatoid factor.
From [1], with permission.

However, as RA is a heterogeneous condition characterised by a wide range of clinical expressivity, different pathological pathways could be predominant in different subsets of the disease.

1.4.1 The adaptive immune system response

The adaptive immune response allows for generation of effector cells with specificity toward a particular pathogen or antigen. It involves mechanisms of cellular and humoral immunity mediated by T and B lymphocytes.

T cells include two main subtypes: CD4+ T helper (Th) and CD8+ T cytotoxic cells. RA has been long considered the prototype of a classic CD4+ Th cells mediated disease. CD4+ Th cells are activated by antigens presented by professional antigen presenting cells (APC) -macrophages, dendritic cells and B cells- through a MHC class II restricted mechanism. Once bound to the MHC class II complex expressed by the APC, the antigen is recognised by a specific T cell receptor (TCR). T cell activation requires a complementary co-stimulatory signal between CD28 expressed on the T cells surface and CD80/86 (B7-1 and B7-2 molecules) expressed on APC. Once activated, CD4+ Th cells secrete a series of cytokines to "help" the activation and function of adaptive and innate immunity.

Two specific CD4+ Th cells subsets exist: type 1 Th cell (Th1), characterised by the expression of T-box transcription factor 21 (TBX21) and the predominant secretion of interferon gamma (INFγ), and type 2 Th cell (Th2), characterised by the expression of trans-acting T-cell-specific transcription factor GATA3 (GATA protein binding 3) and the predominant secretion of the signature cytokine IL-4. These different transcriptional/cytokine profiles confer different functional

properties: Th1 are primary linked to cellular immune responses effected by CD8+ T cells, while Th2 promote humoral immune responses effected by B cells. The immunopathological profile of RA is characterised by a marked shift toward a Th1 immunopathological pattern. [55]

However, new evidence from recent years has added further levels of complexity to the knowledge of the adaptive immune system response, challenging the classic paradigma based on imbalanced Th1/Th2 profile. In particular, a new subtype of CD4+ Th cells has been identified in the last decade, named type 17 Th cell (Th17).

Th17 selectively express the transcription factor RORC2 (retinoic acid receptor-related orphan receptor C2) [56], and are crucial in adaptive defence against extracellular pathogens, as well as in several critical pathogenetic mechanisms of inflammation and autoimmunity. These cells require specific cytokines for their proliferation and function, including IL-1, IL-6, IL-23 and transforming grow factor β (TGFβ), that are abundantly present in the RA synovium. In turn, Th17 secrete a series of inflammatory cytokines such as tumor necrosis factor α (TNFα), IL-6, IL-21, IL-22 and IL-17, which amplify the synovial inflammation and mediate cartilage damage and bone resorption. [57]

Furthermore, it has been discovered that, other than classic CD4+ and CD8+ patterns, T cells can differentiate into a third lineage, $CD4^+$ $CD25^+$ regulatory T cells (Treg). Treg are characterised by the expression of the transcription factor forkhead box P3 (FOXP3), and have a primary role in maintaining immunological self-tolerance. Murine experiments have shown that, by depleting this cell

population, a series of autoimmune disorders could be induced. Conversely, immune-tolerance is restored by transferring adaptive Treg.[58]

Treg counter-regulate the pro-inflammatory function of Th17 and vice versa. Th17 and Treg are therefore in a dynamic balance depending on the local cytokine milieu, including levels of IL-2, IL-6 and TGF-β. When such balance is altered, the maintenance of immune homeostasis is compromised, and pathogenetic mechanisms leading to malignancy and autoimmunity can occur.[59]

B lymphocytes are the main effector cells of the humoral immune system. Following lymphopoiesis in the bone marrow, naive B cells transit to secondary lymphoid organs (lymph nodes, spleen and mucosa associated lymphoid tissue, MALT) for final maturation. Of most importance, the inflamed synovium can become a site for formation of tertiary lymphoid organs, where ectopic germinal centres can develop and function.

In peripheral lymphoid organs, B cell maturation and activation is supported by local cytokines and co-stimulatory molecules derived from resident mesenchymal cells, especially follicular dendritic cells (FDC). A key step in the maturation process is somatic hypermutation in the variable regions of heavy and light chain immunoglobulins and class switching. Eventually, some follicular B cells will differentiate to plasma cells and secrete autoantibodies on antigen exposure, while others will return to a quiescent stage as memory B cells.

As B cells are classic effectors of humoral immune response, the presence of autoantibodies such as rheumatoid factor (RF) and ACPA has long suggested a key role for B cell-mediated immunity in the pathogenesis of RA. However the role of B cells is indeed not restricted to autoantibody production. B cells are efficient

APC and mediators of T cell activation, as well as active producers of pro-inflammatory cytokines, chemokines, adhesion molecules and pro-angiogenetic factors that orchestrate and amplify the immune response. [60]

A population of regulatory B cells (Breg) has also been recognised, which seems to represent a source of inhibitory cytokines such as IL-10 and TGFβ. However, the characterisation of the role of Breg in autoimmune diseases is still underway. [61]

The pivotal role of B cells in the pathogenesis of RA is supported by the therapeutic efficacy of the B cell depleting agent Rituximab. [62]

1.4.2 The innate immune system response

The innate immune response, although not specific, represents the first-line immunological response against biological, chemical or physical insults. Macrophages, neutrophils, dendritic cells (DC), eosinophils, natural killer cells (NK) and mast cells are the main effectors of innate immunity. [63]

Macrophages are supposed to have a primary role in the pathogenesis of RA. They are highly expressed in the inflamed synovium and show a state of activation. Macrophages act as powerful APC and are able to release a high number of pro-inflammatory cytokines and mediators of structural damage: TNFα, IL-1, IL-4, IL-6, IL-10, IL-15, IL-18, IL-32, granulocyte monocyte-colony stimulating factor (GM-CSF), interferon-α and interferon-β (IFNα/β), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and several others. [1] Indirect evidence supporting their pathogenetic role in RA is the consistent observation that synovial sub-lining macrophage number falls in line with effective therapy.[64]

DC function as efficient APC that activate the expansion of clonal CD4+ T cells in the lymph nodes, therefore they have a central role in the modulation of immune tolerance. In RA patients, a high degree infiltration of DC in the synovium has been observed, suggesting in-situ perpetuation of auto-antigen presentation. They contribute to the maturation and differentiation of B-cells as well, and display a high level of pro-inflammatory cytokines expression. [65]

The role of neutrophils in the pathogenesis of RA has been recently highlighted. Neutrophils are abundantly expressed in the synovial fluid of RA patients, and may show several alterations in phenotype and function. Neutrophil activation causes granular release of mediators of cartilage damage, along with up-regulation of pro-inflammatory pathways such as NF-kB. [66,67]

A role in RA immunopathogenesis has been proposed for mast cells in recent years. These cells, classically involved in Th2-mediated inflammation and allergies, participate in other forms of immunity as well. Their number is significantly expanded in RA synovium, where they actively promote the release of vasoactive mediators such as histamine and leukotrienes, cytokines (TNFα, IL-4, IL-5 and IL-6), adhesion molecules, chemokines and degradation enzymes, resulting in the amplification of chronic inflammation and ultimately organ damage. [68] It has been recently demonstrated that mast cells are a source of IL-17, a cytokine that has an important role in RA immunopathogenesis. [69]

Effector mechanisms of innate immunity are activated following the recognition of exogenous or endogenous stimuli by specific receptors called pattern recognition receptors (PRRs). These receptors represent innate sensors able to recognise a repertoire of specific ligands, which can be either microbial

components -pathogen associated molecular patterns (PAMPs)- or endogenous products of cell damage -damage associated molecular patterns (DAMPs)- like nucleic acids, heat shock proteins (HSPs), hyaluronic acid (HA), oligosaccharides, heparan sulfate, glycoprotein gp96, fibronectin, fibrinogen, surfactant protein A, fatty acids. [63,70]

Among PRRs, an increasing interest has emerged for toll like receptors (TLRs) in the recent years. TLRs form a family of trans-membrane receptors expressed on the cell surface and endosome of macrophages, fibroblasts, mast cells, DC, epithelial and endothelial cells. To date, 10 TLRs subtypes have been identified in humans (TLR 1 to 10). It has been proposed that dysregulated TLR signalling may provide a pathway to autoimmunity. Indeed accumulating evidence suggests that DAMPs could be implicated in the auto-antigen recognition and perpetuation of non-infectious inflammation. [70] TLR signalling is dysregulated in animal models of arthritis. [71] An overexpression and enhanced function of TLRs 2, 3, 4 and 7 has been observed in the RA synovium, which is associated with the production of a number of pro-inflammatory cytokines and metalloproteinases.[72-74]

1.4.3 Role of resident cells

Resident joint cells such as fibroblast-like synoviocytes (FLS), chondrocytes, osteoblasts and osteoclasts contribute to maintenance of extracellular matrix and joint homeostasis in the normal synovium. In RA, they tend to acquire a transformed, aggressive phenotype, becoming active participants in the chronic inflammatory response and joint damage.

The central role of FLS in the pathogenesis of RA has clearly emerged over the last decade. Far from being inert structural cells, they contribute to initiation and amplification of the immune response, directly promoting activation of effector cells. [75,76]

The transformation of FLS toward a pathogenic phenotype involves the activation of pivotal downstream signalling cascades such as the mitogen-activated proteins (MAP) kinase system and the transcription factor NF-kB, promoted by cytokine-dependent (especially TNFα, IL-1, IL-6 and IFNγ) as well as cytokine-independent pathways. [77] As a result, FLS acquire somatic mutations of proto-oncogenes and tumor suppressor genes which are similar to those observed in neoplastic transformation. Basically they turn from local homeostatic cells into hyperplastic and dysfunctional cells which become capable of activating immune pathways and promoting inflammatory responses. [75,78]

Once acquired such pathogenic features, FLS become critical in the organization of the inflamed synovium architecture. They actively promote a cell-to-cell interaction with leukocyte and mesenchymal cells, enhancing their activation and function. This cross talk requires the presence of special adhesion molecules, particularly cadherin-11, a trans membrane glycoprotein that facilitates cell-to-cell surface adhesion. The importance of cadherin-11 has been highlighted in murine models of arthritis, where the absence of this protein resulted in a hypoplastic and less inflamed synovium. [79] Several ongoing studies are aimed at identifying markers of FLS activation, and cadherin-11 seems an attractive candidate due to its specificity to the inflamed synovium.

Transformed FLS release a variety of pro-inflammatory cytokines, growth factors, adhesion molecules and angiogenic factors. They are a key source of mediators that promote recruitment, differentiation and activation of Th1 cells, as well as cytokines (IL-6, IL-23, IL-15 and TGFβ) able to influence the expansion and differentiation of Th17 cells. In turn, T-cells can induce the activation of FLS trough direct cell-to cell interactions or via the release of mediators such us IL-17 and IFNγ, generating an intricate loop that contributes to amplify and perpetuate the inflammatory cascade in a bi-directional fashion. [75,76,80]

FLS are also involved in the enhancement of B-cell function: they sustain IL-15-dependent B-cell survival, and promote immunoglobulin class-switching via B cell activating factor (BAFF, also known as B lymphocyte stimulator, BLyS). [81] They are also critical in the organization and function of synovial germinal centres.

There is evidence that FLS are effectors of innate immunity as well. They express TLRs (predominantly TLR 2, 3 and 4) [81] and other receptors involved in the activation of innate immunity responses such as nucleotide-binding oligomerization domain-like receptor (NLR) and protease-activator receptor 2 (PAR2). In-vitro studies demonstrated that they may act as professional APCs after acquiring the capacity of processing and presenting antigens to T cells via an MHC class II-restricted mechanism. [82]

FLS also promote angiogenesis through the production of VEGF and PDGF. [76] Ultimately, activated FLS release matrix degrading enzymes such as MMP-1, A-disintegrin and metalloprotease with thrombospondin-1-like domains (ADAMTS), cathepsin L and aggrecanases, which lead to cartilage destruction. [80] Notably, they

are a source of the receptor activator of nuclear factor κ-B ligand (RANKL) and other molecules directly involved in bone erosions such as dickkopf-1 (DKK-1). [79]

In summary, FLS represent a complex and intricate link between adaptive and innate response, which orchestrate immunopathological pathways leading from synovial inflammation to tissue damage. In consideration of their multipotent pro-inflammatory properties, FLS are becoming an attractive target for new classes of drugs in RA.

Figure 1.2 summarises the main pathways and mediators involved in the complex interplay between immune response, adaptive response and synovial resident cells in RA.

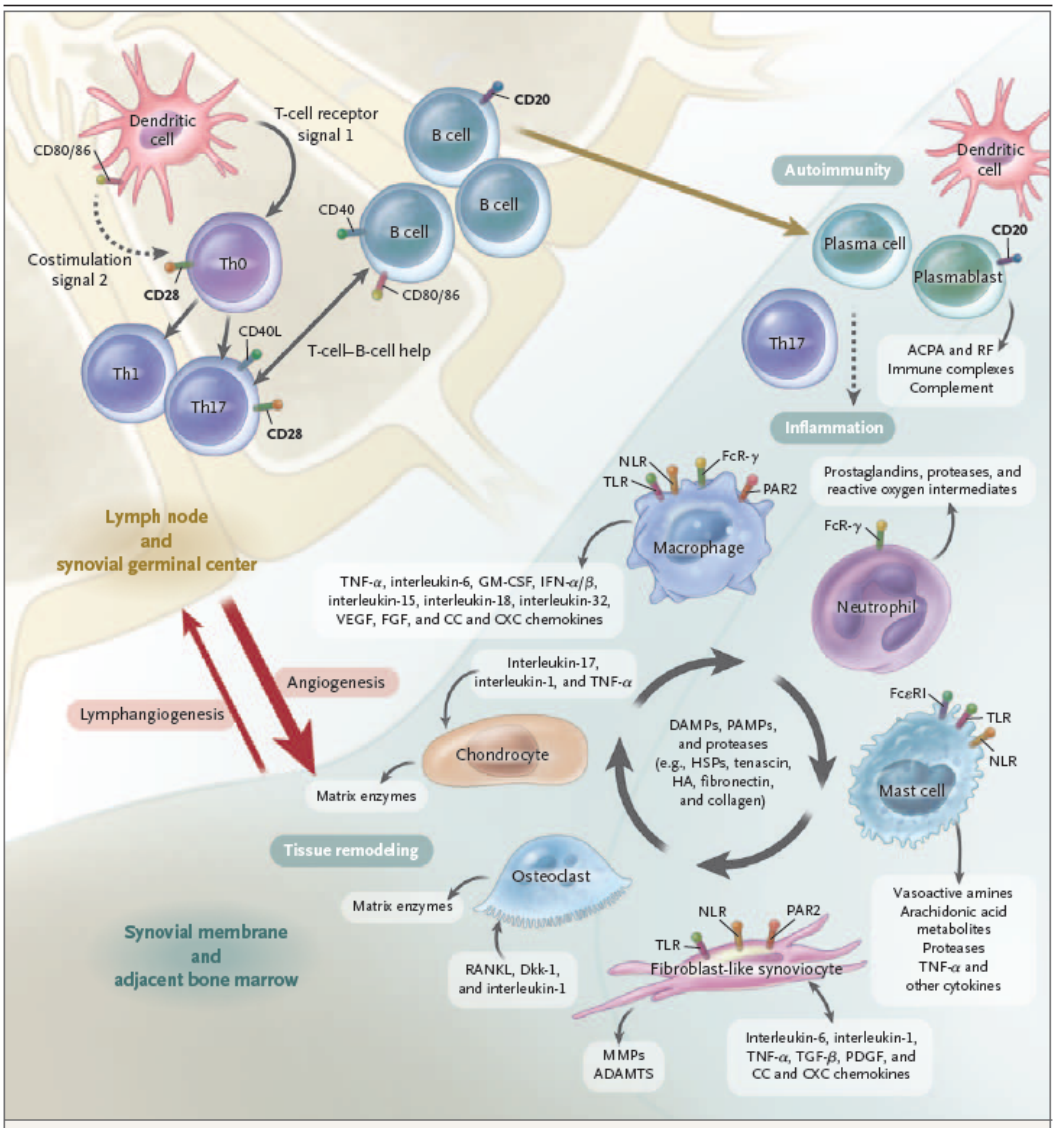


Figure 1.2 : Interplay between adaptive immunity, innate immunity and resident cells in rheumatoid arthritis

In the synovial membrane adaptive and innate immune pathways integrate with resident cells to promote and perpetuate inflammation, tissue remodelling and damage.

Abbreviations: ACPA= anti-citrullinated protein antibodies; ADAMTS= A-disintegrin and metalloproteinase with thrombospondin-1–like domains; CC= CC chemokine; CD40L= CD40 ligand; CXC= CXC chemokine; CSF= granulocyte–macrophage colony-stimulating factor; DAMPs= damage-associated molecular patterns; Dkk-1= dickkopf-1; FcεRI= high-affinity IgE receptor; FcR-γ= Fc receptor γ; GM-FGF= fibroblast growth factor; HA= hyaluronic acid; HSPs= heat-shock proteins; IFN α/β=interferon α/β; MMPs= matrix metalloproteinases; NLR= nucleotide-binding oligomerization domain–like receptor; PAMPs= pathogen-associated molecular patterns; PAR2= protease-activated receptor 2; PDGF= platelet-derived growth factor; RANKL= receptor activator of nuclear factor κB ligand; RF= rheumatoid factor, TGFβ= transforming growth factor β; Th0= type 0 helper T cell; Th1= type 1 T helper cell; Th2= type 2 T helper cell; Th17= type 17 T helper cell; TLR= toll like receptor; TNFα= tumor necrosis factor α; VEGF= vascular endothelial growth factor.

From [1], with permission.

1.4.4 Autoantibodies

A number of autoantibodies can be detected in the serum of RA patients.

These antibodies are directed against different auto-antigens such as circulating immunoglobulins (e.g. RF), circulating proteins (anti-fibrinogen, anti-plasminogen, anti-fibrin), enzymes (anti-calpastatin, anti-enolase, anti-aldolase, anti-glucose 6 phosphate isomerase), components of cartilage (anti-collagen II, IX and XI), citrullinated antigens (anti-cyclic citrullinated proteins antibodies, anti-fibrin, anti-vimentin, anti-filaggrin and several others, forming the class of ACPA antibodies) or nuclear components such as ribonucleoprotein (RNPs) and HSPs. [83]

Autoantibodies carry out their pathogenic activity via formation of immune complexes, activation of complement and recruitment of inflammatory cells, and enhancement of local damage via Fc receptor γ (FcR-γ).

Murine models have shown that some autoantibodies may have a direct pathogenic role in experimental autoimmune arthritis. ACPA, in particular, were capable of contributing to cartilage damage in the collagen induced arthritis (CIA) mouse model.[84] Little evidence exists to support a pathogenic role in humans.

It is possible that, among the several autoantibodies identified in RA, some may represent only an epiphenomenon of chronic inflammation and not be relevant to disease pathogenesis whilst others contribute substantially.

Indeed, RF and ACPA are valuable hallmarks of RA, defining clinically distinct subsets of disease, with important diagnostic and prognostic implications.

RF was first described by Waaler in 1939. [85] RF is an antibody directed against the crystallized fragment (Fc) of immunoglobulin G (IgG), which may exist in different isotype forms: immunoglobulin E (IgE), immunoglobulin M (IgM),

immunoglobulin A (IgA), and IgG. IgM-RF is the isotype routinely measured in clinical assay. It shows good sensitivity but low specificity for RA, as it is found in several other autoimmune diseases, infectious diseases like hepatitis B (HBV), hepatitis C (HCV) and tuberculosis (TB) and in up to 10% of healthy individuals. Testing for IgG-RF and IgA-RF in conjunction with IgM-RF has been shown to improve diagnostic performance. [60,86]

The first ACPA antibody described in RA patients was in 1964 by Nienhuis, who named it antibody perinuclear factor (APF): this was an antibody directed against the perinuclear keratohyaline granules of epithelial cells from healthy human oral mucosa. [87] Subsequently, a large variety of autoantibodies against citrullinated proteins were discovered. Citrullination is the post-translational modification of the amino acid arginine to citrulline by PAD enzyme, which may be induced by environmental factors in genetically predisposed individuals. This is thought to be the 'primum movens' of breach of immune tolerance in RA, as previously discussed. Citrullinated proteins are found in the RA synovium, and ACPA could be produced locally. [88]

The original assay developed for the detection of ACPA, known as the anti-cyclic citrullinated proteins antibodies 1 (anti-CCP1) test, showed limited diagnostic performance, and was followed by a second generation assay, the anti-cyclic citrullinated proteins antibodies 2 (anti-CCP2) test, that was generated from an expanded library of citrullinated proteins. This second test was able to recognise additional citrulline epitopes, resulting in sensitivity and specificity improvement.[89] The sensitivity of ACPA is estimated around 97%, which is

significantly higher compared to RF. [90] A third anti-CCP generation essay is available since 2005, however its practical use remains limited. [91]

Intriguing, studies from blood donors show that the presence of RF and ACPA has been found in almost 50% of RA patients several years before the onset of symptoms, suggesting the existence of an immunological pre-clinical phase of the disease.[92] Magnetic resonance imaging (MRI) and biopsy studies from the joints of individuals who were seropositive for RF and ACPA but had subsequently not developed any clinical manifestation of arthritis, showed a relatively normal synovium, with minor infiltration of T cells. [93] Such observations support the hypothesis that systemic autoimmunity precedes the development of synovitis, implying that a breach in immune tolerance with development of auto-reactive lymphocytes may start in other anatomical sites than the joints. In some individuals this response is then initiated and maintained within the joints themselves, where additional self-antigens could be localised.

Finally, although not diagnostic, RF and ACPA have a positive predictive value (PPV) for the development of RA in early inflammatory arthritis [94-97] and may help to identify patients that may benefit from early intervention. The PPV has been estimated at 4% for RF seropositive and 16% for ACPA. [98]

The presence of these antibodies has also prognostic value in RA patients, as they are important predictors of structural damage and clinical outcome, and this is especially relevant for ACPA. [99-101]

Collectively, these evidences suggest that RF and especially ACPA may play a key pathogenetic role in initiating and perpetuating RA, imprinting the severity and potentially destructive nature of its clinical course.

1.4.5 Cytokines and intracellular signalling pathways

Cytokines play a critical role in the pathogenesis of RA. Particularly TNFα, IL-6 and IL-1 have major pathogenic relevance and represent the targets of current cytokine-blocking armamentarium.

TNFα was the first cytokine to be recognised as a potential therapeutic target in RA. Indeed the pivotal role of this cytokine in RA pathophysiology is strongly supported by the clinical benefits upon its inhibition.

TNFα is produced by activated macrophages and T cells and exerts biologic functions after binding to its receptors: p55 (TNF receptor I, TNF-RI), which is expressed in several human cells, and p75 (TNF receptor II, TNF-RII), specifically expressed in immune system cells. Increased levels of TNFα can be detected in serum, synovial fluid and synovial tissue of RA patients. [102,103] TNFα is a multifunctional cytokine with pro-inflammatory effects on various cells: leukocytes, macrophages, synoviocytes, chondrocytes, and osteoclasts. TNFα has a key role in the induction of other pro-inflammatory mediators, including cytokines, chemokines, adhesion molecules, angiogenic factors, resulting in the enhancement of leukocytes and monocyte recruitment and function, FLS survival and activation, release of cartilage degradation enzymes and activation of RANK/RANKL system. These pathways ultimately lead to the amplification and perpetuation of the inflammatory cascade, cartilage degradation and bone erosion. [104]

IL-6 is a pro-inflammatory cytokine promoting local leukocyte activation, antibody production, neo-angiogenesis and tissue remodelling. Other than local

effects, it has a pivotal role in inducing systemic inflammation such as the release of acute phase reactants, anaemia, fever, fatigue and dysregulation of lipid metabolism. The IL-6 receptor (IL-6R) is composed of 2 subunits: the 80 kiloDalton (kDa) portion, which represents the IL-6-binding portion, and the 130 kDa, which represents the signal-transducing portion. Two forms of IL-6R exist: the membrane and the soluble form, which represent the pharmacological targets of the monoclonal antibody Tocilizumab. Tocilizumab inhibits the biological activity of IL-6 by competitively binding to IL-6R. [105,106]

IL-1 is a key pro-inflammatory cytokine overexpressed in RA. Its biologic effects are counter-balanced by a natural receptor antagonist (IL-1 receptor antagonist, IL-1Ra). In the RA synovium, IL-1 promotes the activation of leukocytes, endothelial cells, resident mesenchimal cells, osteoclasts and chondrocytes. [107] Despite the primary pro-inflammatory role of this cytokine, the clinical benefits of anti-IL-1 monoclonal antibody Anakinra have been proved of modest entity in comparison to TNFα and IL-6R blocking agents. [108]

Within the IL-17 family, which includes several members from IL-17A to IL-17F, IL-17A has recently emerged has a major contributor to the immune-pathogenesis of RA. [109] Il-17A is in fact the archetypal IL-17 cytokine, and in this thesis I will refer to IL-17A as IL-17. The major source of this cytokine is CD4+ Th17 cells, and in minor entity CD8+ cells, NK, monocytes, neutrophils and mast cells. IL-17 synergizes with other cytokines, especially TNFα, IL-1 and IFNγ, to promote the activation of leukocytes, monocytes and resident mesenchimal cells, the synthesis of inflammatory mediators, the release of degradation enzymes and the differentiation of osteoclasts via the RANK/RANKL axis. Once the inflammatory

cascade has been activated, several positive feedback-loop mechanisms contribute to increase the synthesis of IL-17 further. The synovial levels of IL-17 correlate with disease activity and severity.[110,111] Il-17 has became an attractive therapeutic target in several autoimmune conditions, and the IL-17 blocker monoclonal antibody Secukinumab has been recently licensed for use in plaque psoriasis and psoriatic arthritis. [112] Trials for the use of Secukinumab in RA are currently ongoing, however preliminary results are not encouraging. [113]

The differentiation of Th17 cells and the synthesis of IL-17 is dependent on IL-23, another cytokine highly expressed in the synovial fluid and synovial tissue of RA patients. Blocking the IL-23/Th17 axis is therefore an additional attractive therapeutic target. [114]

A major role is also played by cytokines and chemokines predominantly involved in the tropism of B lymphocytes and in the organisation of germinal centres. Within the inflamed synovium, high levels of BAFF are secreted by macrophages, DC, neutrophils and FLS. A BAFF homologue, the proliferation-inducing ligand (APRIL), is released by macrophages, DC and T cells. These cytokines are responsible for B-cell differentiation and activation, antibodies class switch recombination, and B-cell dependent T-cell activation [115-117]

CXC-chemokine ligand 13 (CXCL13), CC-chemokine ligand 21(CCL21) and lymphotoxin β (LTβ), which are mediators critically involved in the formation and function of synovial germinal centres, are also abundantly expressed within the inflamed joint tissue. [104]

Several other cytokines play an active role in RA synovitis: IL-15, IL-18, IL-32, IL-33, GM-CSF, macrophage migration inhibitory factor (MIF) and many others, some of which are currently undergoing testing as potential therapeutic targets.[118-120]

1.5 CLINICAL FEATURES

RA is characterised by persistent synovitis, systemic inflammation, autoantibody production, cartilage and bone destruction, leading to chronic disability and reduced life expectancy. [2] However the disease shows a wide spectrum of clinical phenotypes: some patients have a mild, non-erosive course, whereas others present with severe, rapidly erosive and disabling disease.[121]

1.5.1 Clinical presentation

The typical onset of RA is characterised by progressive, symmetrical joint tenderness and swelling in the metacarpophalangeal (MCPs), proximal interphalangeal (PIPs) and metatarsophalangeal (MTPs) joints. An involvement of the wrists is also very common in the early phase of the disease, meanwhile large joints such as knees, elbows, shoulders, ankles and hips are less frequently involved. Although any joint can virtually be affected, the distal interphalangeal joints (DIPs), the thoracolumbar spine and the sacroiliac joints are normally spared. Peri-articular structures, especially tendons and bursae, are frequently involved. Morning stiffness lasting over 30 minutes with impaired articular function, followed by progressive improvement during the day, is a typical feature of RA. Fatigue is often present. More severe systemic symptoms like fever, profound malaise, weight loss and depression can also be observed.

There is no laboratory test that can confirm or rule out the diagnosis of RA, however abnormal values reflecting systemic inflammation and activation of humoral immunity are usually detected serologically: raised inflammatory markers such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), normocytic normochromic anaemia, thrombocytosis, seropositivity for RF and ACPA [122,123]

An atypical onset occur in about 25% of cases, including monoarticular involvement of a single large joint (knee, shoulder, ankle), predominant peri-articular involvement (tenosynovitis, bursitis), systemic manifestations without joint symptoms, palindromic onset (recurrent episodes of oligoarthritis lasting 1-2 days and spontaneously remitting with no residual radiologic damage). Polymyalgia rheumatica-like onset (involvement of the proximal muscles of the shoulder and pelvic girdle with associated raised inflammatory markers) can be observed in the elderly. [122]

1.5.2 Extra-articular manifestations

Extra-articular manifestations (EAM) of RA can affect various organs. However it may be difficult distinguish them from the comorbidities and the long-term complications associated with RA.

A specific definition for EAM does not exist. Some diagnostic criteria (Malmo criteria) were proposed in 2004 but they are not universally accepted. [124] Partially due to this inconsistency, the prevalence of EAM varies across studies and registers.

In clinical practice, the prevalence of extra-articular features is observed in about 1% of patients or less. However, skin manifestations such as rheumatoid nodules can be detected in up to 30% of patients over the course of the disease. Anaemia, secondary Sjogren's Syndrome and pulmonary complications are also relatively common, ranging from 6 to 10%. [125]

The prevalence of EAM correlates with the duration of disease, although they can virtually occur at any stage, including the early phases of RA. [126]

There are no specific predictors for development of EAM, but they are more frequently associated with male gender, smoking, high levels of inflammatory markers, presence of autoantibodies and the HLA SE. [125]

The presence of EAM is related to worse outcome, including higher disease activity, erosions and disability, and overall increased mortality.[127] A higher risk of cardiovascular events has also been observed. [128]

EAM of RA includes a wide range of cutaneous, pulmonary, cardiac, vascular, ocular, neurological, haematological and renal features.

Among the cutaneous manifestations, rheumatoid nodules are the commonest. [129] These nodules are solitary or multiple subcutaneous granulomatous lesions that preferentially localise in areas of repetitive mechanical pressure such as the elbows, the extensor surfaces of the forearm, the hands and the feet. Nodules can also occur in internal organs, especially lungs, pleura, pericardium, meninges. [130] Accelerated nodulosis has been observed during Methotrexate therapy [131], and recently an association with other therapies including Leflunomide and anti-TNFα agents has been reported. [132] Other typical skin manifestations include cutaneous vasculitis, pyoderma gangrenosum and granulomatous dermatitis. [133]

Pulmonary involvement is common, occurring in 5-10% of RA patients, and represent a major cause of morbidity and mortality: pleural effusions, interstitial lung disease, pulmonary fibrosis, pulmonary nodules, bronchiolitis obliterans and pulmonary hypertension have been observed. [134]

All cardiac structures can be involved in RA, therefore a vast range of cardiac complications has been described: pericarditis, myocarditis, endocarditis, valvular disease, arrhythmias, coronary arteritis, aortitis, ischaemic heart disease. [135] Pericarditis is the commonest cardiac EAM: symptomatic pericarditis has been observed in about 1%–4% of patients, however subclinical pericarditis can be detected on echocardiogram or autopsy in up to 30%–50% of patients. [136]

Ophthalmic manifestations include keratoconjuntivitis sicca (secondary Sjögren's), episcleritis, scleritis, keratitis and retinopathy. Ocular involvement occurs in up to 27% of patients, with secondary Sjögren and scleritis being the commonest manifestations observed. [137] Necrotizing scleritis and peripheral ulcerative keratitis (also known as 'corneal melt') are the most severe ocular manifestations associated with RA, which could lead to visual loss if not promptly identified and treated. [138]

Neurological manifestations of RA can involve both the central and peripheral system. Peripheral nervous system involvement can be secondary to vasculitis, nerve entrapment, amyloid deposition. Mononeuritis multiplex, mediated by vasculitis of vasa nervorum secondary to immune complex deposition, is the most frequent peripheral manifestation. Carpal tunnel syndrome is relatively common and is associated with compression of the median nerve at the wrist level, mainly in consequence of local synovial hypertrophy. [139] A serious complication of long-

standing disease is cervical myelopathy secondary to atlanto-axial subluxation, as the result of local synovitis and bone deformities. [140] Isolated vasculitis of the central nervous system and aseptic granulomatous meningoencephalitis have also been reported, although rarely. [141,142]

Renal involvement is not frequent, mostly represented by glomerulonephritis, interstitial nephritis and nephrotic syndrome as the result of amyloid deposits in long-standing disease. [143]

Hematological EAM are very common, especially chronic anaemia, neutropenia, lymphopenia and thrombocytopenia. [144] Mild to moderate anaemia and thrombocytosis are often observed during acute flares of RA. Finally, Felty's syndrome is a rare condition characterised by polyarthritis, neutropenia and splenomegaly, observed in less than 1% of RA patients. [145]

1.5.3 Natural history of rheumatoid arthritis: disability, comorbidities, mortality

Structural joint damage is observed in 15-30% of patients with early RA. [146] Joint destruction can evolve rapidly: radiographic evidence is present in almost 50% of patients within the first year [147] and in more than 80% of patients within the first 2 years [148]. MRI can identify synovial hypertrophy, bone oedema, and early erosive changes as early as 4 months after symptoms onset. [149-151]

The grade of disability parallels the progression of the joint damage over time. [152] However, loss of independence and loss of ability to work can occur early: it has been estimated that 20-30% of patients become permanently disabled and are forced to stop working during the first 2-3 years. Older age, a physically

demanding job and lower social status are risk factors for premature loss of performance at work. [153]

Disability accounts for a significant proportion of the substantial cost of RA, both at the individual and societal level: absence from work, work restrictions resulting in reduced income, job loss, need for health care, entitlement for socio-economic benefits and disability pensions have a high impact on the indirect financial costs of the disease.[154,155]

RA is associated with comorbidities that may result in worse long-term outcome and reduced life expectancy, with additional individual and societal costs. [156,157] A recent study showed that a persistent inflammatory status was associated with the onset of at least one new co-morbidity during 5 years follow-up period in patients with early RA. [158] The more relevant co-morbidities are osteoporosis, cardiovascular (CV) diseases, malignancies, infections, gastrointestinal (GI) diseases.

Two forms of osteoporosis occur in RA: periarticular osteoporosis around the inflamed joints, and generalized osteoporosis affecting the axial and appendicular skeleton. Periarticular demineralization represents a prominent feature of early RA, and one of its radiographic hallmarks along with erosions and joint space narrowing. Local bone tissue remodelling is the result of reciprocal interactions between osteoclasts, osteoblasts and immune system cells. In fact the inflamed synovium is a source of a number of cytokines and inflammatory mediators that can induce the recruitment, differentiation and activation of osteoclasts. Osteoclastogenesis requires the presence of RANKL and the permissive factor macrophage colony stimulating factor (M-CSF), secreted by osteoblasts and

stromal cells. RANKL binds to its receptor RANK expressed on the surface of osteoclast precursor cells and stimulates their differentiation into mature, functional cells. Raised levels of TNFα, IL-1, IL-6, IL-7, IL-17 in the synovium lead to increased expression of RANKL and M-CSF, and in turn to pathological, accelerated loss of bone mass. [159] Moreover, RA synovium is a source of DKK-1, a physiological inhibitor of bone formation that works inactivating the Wnt-signalling pathway. [160] In addition, the rheumatoid synovium is enriched with cells of the monocyte/ macrophage lineage that, under the influence of appropriate stimuli, can differentiate into pre-osteclasts and ultimately into functional osteoclasts. [159]

Generalized osteoporosis in RA has a multifactorial aetiology. It is related partially to factors intrinsic to the disease -primarily the over-expression of pro-inflammatory cytokines and the activation of pathways of bone resorption- but also to co-factors including reduced mobility and use of corticosteroids.[161] Importantly, a significant amount of bone mass is lost in the early phase of the disease, and the entity of bone loss and associated risk of osteoporotic fractures correlates with the disease activity. [162,163] RA has been incorporated as a major determinant of global fracture risk in the FRAX (Fracture Risk Assessment Tool) algorithm developed by the University of Sheffield in association with the World Health Organisation (https://www.shef.ac.uk/**FRAX**/tool.jsp). A study that included over 30.000 RA patients from the British General Practice Research Database, revealed that the relative risk (RR) for a hip fracture was 2.0 and for a vertebral fracture 2.4. [164]

Patients with RA have a reduced life expectancy compared to the general population, with standardized mortality ratios ranging from 0.87 to 3.0. Ischaemic

hearth disease and ischaemic stroke represent the major contributors to this excess mortality. [165-167] It has been estimated that the increased CV risk observed in RA is comparable to that associated with type 2 diabetes. [168] [169] These high rates of CV morbidity and mortality cannot be entirely explained by traditional CV risk factors. RA is characterised by endothelial dysfunction, lipid metabolism alterations and accelerated atherosclerosis, and systemic inflammation and immune dysregulation have a key role in determining this pro-atherogenic status. Indeed the concept that inflammation is a key component of atherosclerosis has been explored by a number of studies [170-174] supporting the concept that atherogenesis is an inflammatory-driven disorder. It is also acknowledged that sustained systemic inflammation represents an independent predictor of CV events in the general population. [175, 176]

Patients with RA have twice the risk of developing congestive heart failure compared to the general population [177], and mortality from congestive hearth failure is higher in RA in comparison with non-RA individuals. [178]

Malignancies represent the second commonest cause of death in patients with RA. A meta-analysis by Smitten *et al* showed a two-fold increased risk of lymphoproliferative disorders, especially Hodgkin's lymphoma, as well as lung cancer and melanoma. [179,180] When evaluating neoplastic risk in the RA population, it is difficult to discriminate between the intrinsic effects of RA and confounding factors such as immunosuppressive drug exposure [181]; however, recent evidence suggests that persistent immune system activation rather than treatment is a major determinant. [182]

Infections are an important cause of morbidity and mortality in RA: pulmonary infections, urinary tract infections, sepsis, osteomyelitis, cellulitis and septic arthritis are the most frequently observed. [183,184] This increased risk is, at least in part, related to intensive and prolonged immunosuppressive therapy. Furthermore, RA per se is characterised by intrinsic dysregulation of the immune system that may predispose to infections. This is also suggested by the fact that the infective risk is higher in patients with more active disease. [185]

Finally, RA has long been strongly associated with GI events, particularly in the upper tract, ranging from minor manifestations like dyspepsia and nausea to more severe organic conditions such as esophagitis, gastritis, duodenitis, gastric and duodenal ulceration and perforation. [185] RA showed a 50% increased risk of death related to GI events from the Arthritis Rheumatism and Aging Medical Information System (ARAMIS). [186] A large epidemiological study conducted on hospitalized patients found that RA subjects presented a 5 fold higher mortality from GI causes than the non-RA subjects. [187] This increased risk has been historically related to use of non-steroidal anti-inflammatory drugs (NSAIDs). Indeed, a progressive decline in the incidence of GI complications in RA patients - estimated about 0.5% per year by 2000 - has been observed in coincidence with a reduced use of NSAIDs after the introduction of biologic disease-modifying anti-rheumatic drugs (bDMARD), the development of cyclooxygenase 2 (COX-2) inhibitors and the increasing use of proton-pump inhibitors. [185] GI complications intrinsically related to the pathogenesis of the disease, such as vasculitis and amyloid infiltration of the gut have also been described, although these rarely lead to clinically detectable symptoms.[188]

Persistently active disease, presence of comorbidities and extra-articular features are the most important factor risks associated with reduced survival in RA patients compared to the general population.[157] Advances in the treatment of RA in recent years, such as early intervention, target to treat strategy and introduction of biologic drugs have significantly modified the natural history of the disease and improved outcome and quality of life. [189] Also, patients who respond to methotrexate have only a slightly increased mortality risk compared to general population, meanwhile the non responders present a 4 fold increased risk.[190]

Nonetheless, data from a population-based incidence cohort of RA patients from the Mayo Clinic, Rochester, Minnesota, published by Gonzalez and colleagues in 2007, disappointingly showed that the mortality rate for RA patients has remained unchanged over the past four decades, and the survival gap between general population and RA population has even widened. [191] However, the authors highlight that their findings cannot be extrapolated to RA patient after 2000, when the use of bDMARD and more intensive treatment strategies were introduced.

1.6 RATIONALE FOR EARLY DETECTION AND TREATMENT OF RHEUMATOID ARTHRITIS

1.6. 1 Advantage of early diagnosis and treatment: the 'window of opportunity'

Early identification and treatment of RA represent an effective chance to improve outcome, with a considerable reduction of individual and socio-economic costs. Several studies have showed that prolonged symptom duration and delayed treatment result in more severe radiological damage and a lower chance of

achieving clinical remission. [192] Even a relatively short delay in starting treatment may have a negative impact on disease progression. [193] Importantly, the clinical, radiological and functional benefits obtained treating the disease earlier appears to be sustained over time.

Indeed, the early phase represents a unique 'window of opportunity' for effective treatment. [194] Van der Heijde and colleagues randomised 238 patients with RA for less than the 12 months to receiving either NSAIDs or conventional syntetic disease-modifying antirheumatic drugs (csDMARD); after 12 months, the group that had received csDMARD experienced more significant clinical improvement compared to the NSAIDs group. [195] Stenger and colleagues compared the effect of aggressive versus step-up treatment on radiographic progression in 228 patients: after 2 years follow-up, the radiographic progression was significantly lower in the group treated aggressively at onset. [196] A recent meta-analysis of 12 studies found that, in patients with a diagnosis of RA standing less than two years, a nine month delay in starting treatment resulted in a 33% more severe radiological damage. [197] A meta-analysis of 14 randomised controlled trials (RCTs) found that shorter disease duration was the strongest predictor of clinical response to csDMARD therapy, with the best response observed in patients presenting with less than one year symptoms. [198] Recently, the PROMPT (PRObable rheumatoid arthritis: Methotrexate versus Placebo Treatment) study compared early treatment versus placebo in patients with inflammatory polyarthritis not fulfilling classification criteria for RA, showing that a lower number of patients in the treatment group progressed to RA over time. [199]

To avoid missing the window of opportunity, a critical point is the early recognition of symptoms by primary care physicians and the rapid referral to rheumatology centres, as well as the institution or pathways for rapid access to them. A survey from 2009 and 2010 showed that the median delay from symptom onset to patients seeing a rheumatologist across 10 European centres was 24 weeks, meaning that a significant proportion of patients were falling outside the therapeutic window of opportunity. [200] The establishment of early arthritis clinics (EACs) has significantly improved this shortfall. The aim of EACs is to allow rapid referral, quick assessment and prompt start of treatment for patients with suspected inflammatory arthritis. The time between symptoms onset and first rheumatological evaluation is on average three months shorter within EACs compared with standard clinics, and this translates into an immediate and sustained better clinical outcome with cost saving over the long run (e.g. higher rate of patients achieving remission, less requiring biologic drugs, less sustained disability). [201]

Another aspect of critical importance is whether the advantage of earlier intervention is indefinite or there is a certain point when the window of opportunity closes. It looks like the relationships between symptom duration and favourable outcome is not linear and that a time limit is reached after which the benefit of starting treatment earlier is missed. Data from the Leiden cohort shows that the window of opportunity seems to start closing at 14.9 weeks. Overall the Leiden experience suggest that there is a period, within approximately the first 6 months of symptom onset or potentially an even shorter period, in which

csDMARD treatment must be started in order to achieve better long-term outcomes. [202]

1.6.2 Defining early rheumatoid arthritis: how early is early?

Since timing is crucial for optimizing outcome, defining early RA is not a purely semantic matter, but has got important practical implications.

A major issue for the definition of early RA is the heterogeneous definition of disease 'onset', which in some studies refers to the time of initial symptoms, in others to the time patients seek medical attention, or to when a formal diagnosis is made. Another critical issue is that recognizing the earliest symptoms of RA is challenging for patients, as some early symptoms such as morning stiffness and fatigue are poorly defined and may remain underestimated for several weeks or months. [203]

NICE (National Institute for Health and Care Excellence) recommend that patients with suspected inflammatory arthritis should be referred urgently for assessment if symptoms have been present for more than six weeks and any of the following apply:

- swelling of two or more joints is present;
- the small joints of the hands or feet are affected;
- there is a positive MCP or MTP joints squeeze test (pain produced by gentle pressure across the MCP or MTP joints).

Other features that should raise suspicion of inflammatory arthritis include:

- early morning joint stiffness for more than 30 minutes;
- raised inflammatory markers.

As mentioned in the previous paragraph, the establishment of EACs has represented a fundamental strategy to ensure rapid referral and rapid assessment of patients with suspected early inflammatory arthritis.

Currently, the accepted definition of early RA is a disease not exceeding 12 months duration. [204,205] However the notion of 'early' is becoming earlier. A study comparing clinical and radiological outcomes in two groups of patients who received csDMARD treatment within either 12 weeks or 12 months after symptoms onset, showed that the earlier group performed significantly better. [206] Similar findings have been confirmed in other studies [207,208], highlighting the importance of timing as a major determinant of therapeutic success. There is accumulating evidence that the first 12 weeks from onset represent a distinct pathological phase, which has led to the concept of 'very early' RA.

The question is whether unique pathological processes operate during the earliest stages. Raza et al found that the synovial fluid of patients with RA within the first 12 weeks is characterised by marked absence of IFN-γ and predominance of IL-4 and IL-13, IL-2, IL-15, IL-17, fibroblast growth factor and epidermal growth factor, defining a transient Th2 response that subsequently shifts towards a persistent Th1 response. [209] McInnes et al recently postulated the existence of a predominant cytokine pattern in different phases of the disease, namely a pre-clinical/early transient pattern (IL-6, IL-21, IL-23, IL-17 predominant), followed by a pattern reflecting transition to chronicity (TNFα, IL-6 predominant) and ultimately a permanent pattern reflecting persistence of immune activation and damage, introducing the concept of 'stage-dependent pathogenesis'. [210]

However, as data on synovial pathology within the first months after the onset of symptoms is scarce, currently the concept of 'early arthritis' represents a purely clinical definition rather a pathobiological entity.

The question remains whether the existence of a window of opportunity is based on underlying specific immune pathobiology in the first weeks or months from disease onset. Herein I will explore if there are any specific characteristics that makes early RA a distinct pathobiological as well as clinical entity, and how histological and clinical characteristics are mutually related in this crucial phase of the disease.

1.6.3 From 1987 to 2010 classification criteria for rheumatoid arthritis

Delays in the recognition and treatment of RA may be related to the lack of established criteria to make a definite diagnosis. In fact, diagnostic criteria for RA do not exist and diagnosis is based on physician discretion/expert opinion.

Classification criteria have been developed in order to minimize misclassification and make cohorts homogeneous in clinical and epidemiological studies. The set of classification criteria developed in 1987 by the American College of Rheumatology society (1987 ACR criteria) is displayed on Table 1.1. [129]

1. Morning stiffness
2. Arthritis in 3 or more joints
3. Arthritis of hand joints
4. Symmetrical arthritis
5. Rheumatoid nodules
6. Rheumatoid factor
7. Radiographic changes (erosions)

Table 1.1: The 1987 American College of Rheumatology revised criteria for rheumatoid arthritis

Four out of the seven criteria must be satisfied, with the 1-4 criteria to be present for a minimum of 6 weeks. [129]

These criteria perform poorly in early RA. [211] This is not surprising, considering that they were derived from patients with over 7 years disease duration. At least two of the seven criteria -presence of rheumatoid nodules and erosions- are typically observed in longstanding rather than early disease. When applied to patients with < 1 year disease duration, sensitivity decreased to 81% compared to 91% observed in patients with longer disease duration. [129]

The need to identify patients at an early phase led to development of new classification criteria proposed by ACR and EULAR in 2010 (Table 1.2).[4] These criteria have been constructed through two phases, as a combination of data-driven and expert-driven approaches: in the first phase, Methotrexate (the commonest csDMARD used for the treatment of RA) prescription during the first year after diagnosis was chosen as an indication of RA; in the second phase, the opinion of experienced clinicians was used to reach a consensus for the final criteria set.

Joint involvement	Score
1 large joint	0
2-10 large joints	1
1-3 small joints	2
4-10 small joints	3
>10 small joints	5
Serology	
RF (-) and anti-CCP (-)	0
Low RF (+) or anti-CCP (+)	2
High RF (+) or anti-CCP (+)	3
Acute phase reactants	
Normal ESR and CRP	0
Abnormal ESR or CRP	1
Duration of symptoms	
<6 weeks	0
≥6 weeks	1

Table 1.2: The 2010 ACR/EULAR classification criteria for rheumatoid arthritis
The criteria apply to patients with: 1) evidence of clinical synovitis (at least one swollen joint) 2) synovitis not better explained by another disease. Rheumatoid arthritis is defined by a total score ≥ 6. Joint involvement refers to any swollen or tender joint on examination, which may be confirmed by imaging evidence of synovitis. Distal interphalangeal joints, first carpometacarpal joints, and first metatarsophalangeal joints are excluded from assessment. Large joints refer to shoulders, elbows, hips, knees, and ankles. Small joints refer to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists. Negative refers to international units (IU) values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but three of less times the ULN for the laboratory and assay; high-positive refers to IU values that are more than 3 times the ULN for the laboratory and assay. Where antibody titre is only available as positive or negative, a positive result should be scored as low positive. Normal/abnormal is determined by local laboratory standards.
Abbreviations: RF= rheumatoid factor; anti-CCP= anti-citrullinated protein antibody, ESR= erythrocyte sedimentation rate; CRP= C-reactive protein. [4]

Major differences between the new and the old set criteria are: the removal of rheumatoid nodules; the removal of erosions (highly specific for the diagnosis of RA but not helpful when aiming to identify the disease at an early stage [146]); the removal of morning stiffness due to its lack of specificity; a higher importance attributed to laboratory tests, including incorporation of raised inflammatory markers and presence of anti-CCP antibodies and RF antibodies. Seropositivity is not just counted as a dichotomic parameter, as the autoantibody titre is also considered, with higher levels resulting in higher scores.

Overall, the 2010 ACR/EULAR criteria allow more rapid identification of RA patients compared to the 1987 criteria. However, due to lower specificity, issues related to potential misclassification and over-diagnosis have been raised. [212] Importantly, despite improving ability to identify more patients at an earlier phase, they have limited discriminative value to differentiate potentially destructive from non-erosive, mild disease. [212,213] In particular, it has been observed that the discriminative power for development of erosions in 10 years is only slightly better than the 1987 ACR criteria. [214] Therefore, extra efforts are needed to identify accurate clinical and molecular biomarkers to predict the clinical course of early inflammatory arthritis.

1.7 THERAPEUTIC STRATEGIES FOR RHEUMATOID ARTHRITIS

1.7.1 Aims of treatment

The aims of the treatment of RA have been identified as: symptom control, prevention of joint damage, improvement of quality of life and maintenance of ability to function. [215]

The ultimate goal should be the achievement of sustained clinical remission, which has been defined as "absence of articular and extra-articular inflammation and disease activity".[216] With the introduction of new drugs and optimisation of therapeutic strategies, this is now a realistic goal and should be aimed for most patients, particularly for those identified at an early stage.

However, no universally accepted definition of remission currently exists and remission criteria vary from study to study, making it difficult to compare remission rates across different cohorts.[217,218] In particular, it is not clear if remission should incorporate absence of clinical signs of synovitis, or absence of surrogate markers of disease activity such as inflammatory markers or radiological progression. It has been observed that patients could develop radiographic erosions in the hands and feet over 2 to 5 years after achievement of persistent clinical remission, suggesting that clinical definition of remission may not be stringent enough to ensure absence of disease progression over time.[219]

Indeed, a high proportion of patients considered to be in clinical remission -no matter how stringent these criteria are- shows evidence of residual inflammatory activity by using sensitive imaging technique such as ultrasound (US) or MRI, and this sub-clinical synovitis correlates with ongoing structural damage. [220,221] Therefore, the question arises whether a modern definition of remission should incorporate the notion of imaging remission as well. [222]

Finally, the very ultimate goal should be drug-free remission, which refers to achievement of a sustained state of remission with medications no longer required. From the analysis of several studies, it has been observed that only a small proportion of early RA patients (9%–15%) may be able to achieve this goal.

Among them, up to 45% will require a re-introduction of medications over time, and regaining a satisfactory control of the disease may be more difficult at that point. [219]

Importantly, in order to optimise therapeutic strategies and evaluate achievement of therapeutic goals, baseline status and progresses over time must be objectively measurable, and the measurements reproducible. Disease activity should be formally assessed and documented at onset and, once commenced on treatment, close monitoring of patients and regular assessment of response is crucial.

1.7.2 The Treat to Target strategy

Treat to Target (T2T) is generally defined as a treatment strategy tailored to the individual patient, in which the achievement of an objective outcome measure is used as a goal (the "target") to monitor therapeutic response and guide adjustment of therapy.

T2T that was originally introduced in the field of diabetes care, to design clinical trials focusing on a standardized and objective therapeutic target (the level of glycosylated haemoglobin). Subsequently, this concept spread to other areas of medicine, such as hypertension and lipid metabolism.

Over the past 10-15 years, T2T has been adopted in the management of RA as well. [223] In 2010, ten recommendations were published as the result of the International Treat to Target Initiative for the use of T2T in RA (Table 1.3).

1. The primary target for treatment of rheumatoid arthritis should be a state of clinical remission
2. Clinical remission is defined as the absence of signs and symptoms of significant inflammatory disease activity
3. While remission should be a clear target, based on available evidence low disease activity may be an acceptable alternative therapeutic goal, particularly in established long-standing disease
4. Until the desired treatment target is reached, drug therapy should be adjusted at least every 3 months
5. Measures of disease activity must be obtained and documented regularly, as frequently as monthly for patients with high/moderate disease activity or less frequently (such as every 3–6 months) for patients in sustained low disease activity or remission
6. The use of validated composite measures of disease activity, which include joint assessments, is needed in routine clinical practice to guide treatment decisions
7. Structural changes and functional impairment should be considered when making clinical decisions, in addition to assessing composite measures of disease activity
8. The desired treatment target should be maintained throughout the remaining course of the disease
9. The choice of the (composite) measure of disease activity and the level of the target value may be influenced by consideration of co-morbidities, patient factors, and drug-related risks
10. The patient has to be appropriately informed about the treatment target and the strategy planned to reach this target under the supervision of the rheumatologist

Table 1.3: Ten recommendations on treating rheumatoid arthritis to target
From [224], with permission.

Several RCTs and cohort studies have provided evidence that a T2T strategy ensures achievement of superior outcome compared to standard care. [223]

The modality of monitoring the disease activity must be reliable and sensitive to change, reproducible and feasible for use in clinical practice. Several points remain elusive, and particularly what is the best target to set: should we aim at low disease activity or clinical remission? should imaging remission be formally pursued? [225] and again: what is the ideal frequency of monitoring for achievement of target? should the frequency of evaluation depend on the level of disease activity, so patients with more active disease may require more frequent assessments? should the set target take into account the stage of the disease as well (e.g., it will it be less realistic to pursue formal remission in patients with long standing disease compared to whom with early disease)?

Given the discrepancy between achievement of clinical remission and presence of ongoing active synovitis detected by US, and considering the evidence that this residual activity has a significant impact on future structural and functional outcomes [220,226], the concept that targeting therapy to imaging measures may be superior than targeting to clinical measures only has recently emerged. [227] On the basis of this assumption an international network of ultrasonographers and rheumatologists have instituted the Targeted Ultrasound Initiative (TUI) group. One of the initiatives undertaken is a multicentre study, the Targeted Ultrasound in Rheumatoid Arthritis (TURA) study, in which patients with RA will be randomised to target therapy aiming to the achievement of either both clinical remission and US remission or clinical remission only, to assess whether the suppression of US activity translates into a better outcome. [227]

Very recently the results of the TaSER (Targeting ultrasound remission in early rheumatoid arthritis) study have been published, where 111 newly diagnosed RA patients (< 1year symptom duration) were randomised to csDMARD step-up strategy aiming to either low disease activity or US remission (defined as power doppler joint count ≤ 1). The study showed that the group aiming at US remission required greater intensity of csDMARD therapy, in keeping with the assumption that US remission is more stringent remission. However this was not associated with superior clinical, functional or health-related quality of life outcomes at the end of the 18 months follow-up period. [228] The authors postulate that one of the reasons could be the fact that both populations had an excellent overall response, and this may have limited the power of the study to detect small size inter-group differences. Another possible explanation could be that the follow-up period was too short to detect differences, which could have emerged over much longer follow-up periods.

The ARCTIC trial, where 122 patients with early RA were randomised to an ultrasound tight control strategy targeting clinical and imaging remission and 116 were randomised to a conventional tight control strategy targeting clinical remission, also failed to show the added value of US to clinical driven T2T treatment strategy. [229]

Despite the several theoretical and practical issues implicated, the T2T represents an undisputed revolutionary concept in its linear goal-driven approach: setting a specific target, monitoring changes using a standardized outcome measure, and adjusting therapy accordingly.

1.7.3 A paradigm change: from "pyramid model" to "tight control" approach

For years, the management of RA has been based on a stepped-up approach (the so called "pyramid model"), meaning an initially soft pharmacological regime followed by progressive escalation of treatment to achieve disease control.

NSAIDs and steroids were the initial drugs administered to control joint symptoms, while csDMARD were usually added later in the course of the disease. This approach was based on the assumptions that NSAIDs have a relatively safe profile, meanwhile csDMARD were considered extremely toxic drugs, and RA regarded as an overall benign, non life-threatening disease. [230]

Over the last decade, after these postulations have been proved incorrect and new concepts such as the importance of early identification and treatment of RA have emerged, a dramatic paradigm change has occurred. This translates into the so-called "inverted pyramid" approach (meaning aggressive therapy to start with, followed by gradual tapering once the therapeutic target has been achieved) in order to get early control of inflammation and prevent rapid disease progression. Importantly, the modern management of RA implies not only early intensive treatment but also a closer monitoring of patients, allowing prompt escalation of therapy whenever the therapeutic targets are missed. This strategy is defined "tight control". [231]

Evidence of improved outcomes with the adoption of a tight control strategy has been provided by several studies, such as the Behandel Strategieën (BeSt)[232], the Computer Assisted Management for Early Rheumatoid Arthritis (CAMERA)[233] and the Tight Control of Rheumatoid Arthritis (TICORA)[234] studies.

TICORA, in particular, is a randomised controlled trial specifically designed to compare the superiority of an intensive treatment group versus a standard treatment group in patients with RA and disease duration up to five years. One year after commencement of treatment, remission rates were significantly higher in the intensive therapy group versus the standard care group (65% vs 16%). Similarly, radiographic progression, physical function and quality of life were superior in the tight control group, at no additional costs. Importantly, when the intensively treated group was switched to standard care after 18 months, the initial benefits were lost.[234]

These studies deliver an important message to clinicians: optimal therapeutic strategy and intensity of treatment is not less important than the effectiveness of the drug armamentarium to increase the chances of success in RA management.

A fundamental question remains unanswered though: whether an intensive treatment strategy is necessary for all patients with early RA, or whether for patients at lower risk of disease progression the pursuit of stringent treatment targets results in no net gain but actually in exposure to unnecessary risk related to potential treatment toxicity. Answering this question will be difficult until our ability to stratify patients according to prognostic categories remains limited.[225]

1.7.4 An overview of current therapeutic options

1.7.4.1 Symptomatic drugs

NSAIDs and COX-2 inhibitors are effective in relieving the symptoms of RA, especially joint pain and stiffness. A limitation in the use of these drugs is represented by the lack of ability to interfere with the disease progression and

further by their unfavourable safety profile. NSAIDs use has been historically associated with GI events in RA patients. While the addition of gastro-protective agents or the use of COX-2 selective drugs have significantly reduced the rate of GI complications, both NSAIDs and COX-2 inhibitors have been associated with increased cardiovascular risk, especially fluid retention, exacerbation of hypertension, renal impairment and thrombotic events. Therefore, their use should be limited to the shortest treatment duration possible and after gastrointestinal, cardiac and renal risk have been carefully evaluated at the individual level.[148,235]

1.7.4.2 Glucocorticoids

Despite major treatment advances over the last two decades, particularly the introduction of biologic agents, steroids remain a milestone in the treatment of RA after 65 years of use. [236]

Systemic glucocorticoids, and especially oral prednisolone (PRED), are widely used for the short-term relief of signs and symptoms of RA. They have an important role in establishing control of synovitis particularly in the early phase of the disease and as a "bridging therapy" until csDMARD reach maximum therapeutic effect. Systemic or intra-articular steroids are also helpful in the management of rheumatoid flares.

A Task Force instituted by EULAR with the aim of updating guidelines for the management of RA, recommends that low-dose glucocorticoids should be considered as part of the initial treatment strategy in combination with one or more csDMARD. [237] There is in fact data supporting that the use of steroids, either initially at high dose followed by rapid tapering (e.g. COBRA (Combinatietherapie

Bij Reumatoide Artritis) regimen [238]) or at very low doses over two years [239], may have a disease-modifying effect, showing a beneficial role in retarding radiographic progression. [240-243] It has been observed that the long-term use of low dose of steroids, especially when administered early in the course of the disease, may slow by at least 50% the magnitude of radiographic progression. [244] Additionally, long-term follow-up studies show that treatment regimens which include steroids in the early phase may have a sustained positive impact on the disease course after their discontinuation. [238,245-248]

The main limitation in steroid use is related to side effects depending on cumulative dose -hypertension, obesity, osteoporosis, diabetes, avascular osteonecrosis, cataracts, glaucoma- and therefore long-term use should be avoided. [148,215] It is important that adequate monitoring of potential side effects and specific preventive measures are in place[249-251]. The EULAR Task Force recommends limiting steroid use to a maximum of 6 months, ideally tapering the dose at earlier time points. [237]

1.7.4.3 Conventional synthetic Disease-Modifying Antirheumatic Drugs

Early initiation of therapy with csDMARD, ideally within one month from the diagnosis, is crucial for optimal management of RA.

By definition csDMARD are able not only to control signs and symptoms of RA, but also to retard the progression of the disease. [235] The potential risks associated with csDMARD therapy are acceptable with long-term use, and overall preferable to those related to persistently active, untreated RA. [252]

Unless contraindicated, the current EULAR recommendation is that Methotrexate (MTX) should be part of the first treatment strategy in all patients

with early RA. [148,237] MTX indeed remains the “anchor” drug in the treatment of RA [253], used in monotherapy or in combination with other csDMARD or bDMARD.

MTX monotherapy, with or without steroids, can lead to achievement of low disease activity in about 25–50% of patients with early RA within 6–12 months. [254-256] Notably, some RCTs have shown that MTX monotherapy is comparable to bDMARDs in RA when commenced in the early phase of the disease. [257,258]

Potential side effects of MTX include liver toxicity, skin nodulosis, acute interstitial pneumonitis (that could be potentially fatal), lung fibrosis, cytopenia, mouth ulcers, alopecia, nausea, vomiting, general sickness, and increased risk of infections. However, an acceptable long-term safety profile for this drug has been demonstrated. [259] Important aspects of the use of MTX include dose optimisation and route of administration (oral, intramuscular or subcut) [260], concomitant optimal use of folic acid [261], and recognition that the maximum therapeutic effect is obtained for each dose level after an average of 4–6 weeks [255,257]. In this respect, it is recommended that MTX should be maintained at a sufficient dose and for sufficient time (at least 8 weeks) before progressing to potentially more intensive therapies. [237]

There is good evidence for a disease modifying effect for Sulfasalazine (SSZ) and Leflunomide (LEF) as possible alternative to MTX, meanwhile the evidence is lower for Hydroxychloroquine (HCQ), Cyclosporine, Azathioprine, Penicillamin and oral gold. [215]

Due to its favourable safety profile and ability to improve symptoms and some objective measures of inflammation, HCQ is commonly used for the management of RA, alone in disease perceived as mild and unlikely to progress, or in association

with MTX. SSZ is also frequently used in early or established RA, particularly in association with MTX in those forms of RA perceived as more severe and likely to evolve toward persistent or erosive disease. LEF is considered a good alternative to MTX, with a comparable safety profile. [215,235] Of note, SSZ and HCQ are compatible with conceiving and pregnancy, while MTX and LEF are associated with teratogenicity and require concomitant contraceptive measures/discontinuation if conception is desired. [262]

Whether csDMARD combination therapy is superior to monotherapy, and especially whether the combination of MTX with other csDMARD in the early phase of the disease is superior to MTX alone, is debated. In clinical practice, csDMARD combination therapy is very common, although data are conflicting. Some RCTs showed that combination of MTX with SSZ was not superior to single drug treatment. [263,264] Nonetheless, reports from RCTs in early RA such as COBRA [238,265] and FIN-RACo [266], demonstrated clinical and radiological superiority of combination therapy compared to step-up approach.

A milestone study from O'Dell suggested that "triple therapy" –combination of MTX, SSZ and HCQ– is superior to MTX plus SSZ or MTX plus HCQ to achieve clinical remission in established RA, at no cost of more side effects. [267] In a recent review of current therapeutic strategies for early RA, Sethi and O'Dell found that MTX monotherapy is normally able to achieve therapeutic target in over one-third of patients; when MTX on its own fails this target, triple csDMARD therapy will result in control of another one-third of the patients; the remainder of patients will require biological therapies. [268]

1.7.4.4 Biologic Disease-Modifying Antirheumatic Drugs

In the last decade, there have been significant improvements in the treatment of RA. One of the most important advances has been the development of biologic DMARD (bDMARD or biologics), genetically engineered proteins derived from either murine or human genes. These drugs differ from traditional medications used in the treatment of RA as they target specific components of the immune system that play a key role in activating and perpetuating the inflammatory cascade.

Biologic drugs are used in patients with moderate and severe RA that have not responded adequately to initial treatment with csDMARD (about 30–40% of patients). [269]

The first bDMARD approved for the treatment of RA were inhibitors of TNFα, which were first launched in 1998 [270]. Currently five anti-TNFα are licensed for clinical use in RA: Infliximab, Etanercept, Adalimumab, Certolizumab pegol and Golimumab.

Infliximab is a chimeric (human and murine) IgG1 anti–TNFα antibody administered intravenously, which binds with high affinity to both the soluble and the membrane TNFα and inhibits the interaction between the cytokine and its receptor. [270] Etanercept is a fusion protein consisting of the extracellular region of two human p75 TNFα receptors coupled to the Fc portion of a monoclonal human antibody, administered subcutaneously. Unlike other TNFα inhibitors, Enbrel targets TNFβ (lymphotoxin) as well; another important pharmacological difference, which derives from its biomechanical structure, is that Etanercept is unable to fix complement and lyse cells that express TNFα on their surface. [271]

Adalimumab is a recombinant, fully humanized monoclonal anti–TNFα antibody given subcutaneously. [270] Certolizumab pegol is a pegylated anti-TNFα consisting of a fragment antigen-binding (Fab) attached to a 40 kilo-Dalton polyethylene glycol (PEG) moiety. It is administered subcutaneously every two weeks. The attachment of PEG to the Fab modifies the half-life and may contribute to the preferential distribution of the drug to inflamed tissues. Moreover, Certolizumab lacks the Fc region and so it does not induce complement or antibody-dependent cell-mediated cytotoxicity which has been observed in vitro with other anti-TNFαs. [272] Finally, Golimumab is the last approved human monoclonal antibody to TNFα and is administered subcutaneously at monthly intervals. [273]

Across a large number of trials and registries, the efficacy and safety profile of the five currently available anti-TNFα drugs is comparable [274], however head-to-head studies are scarce. In most of the RCTs, these agents were studied in comparison or in combination with MTX.

At present, the combination of MTX with TNFα blockers appears to provide good therapeutic effect, both in terms of clinical efficacy and prevention of radiological progression. This has emerged from several trials, such as the 'Active-Controlled Study of Patients Receiving Infliximab for the Treatment of Rheumatoid Arthritis of Early Onset' (ASPIRE) [275], the 'Trial of Etanercept and Methotrexate with Radiographic Patients Outcome' (TEMPO) [258], the 'Efficacy and Safety of Adalimumab and Methotrexate versus Methotrexate Monotherapy in Subjects with Early Rheumatoid Arthritis' (PREMIER) [257] and the 'Comparison of Methotrexate Monotherapy with a Combination of Methotrexate and Etanercept in Active, Early, Moderate to Severe Rheumatoid Arthritis' (COMET). [276]

Unfortunately, approximately 30–40% of patients on anti-TNFα continue to have active disease. Among them, some do not respond from the very beginning (primary failure), while other show an initial response but will loose it subsequently (secondary failure). [269]

Access to anti-TNFα agents vary largely across countries depending on national and local regulatory bodies. In some countries the standards for initiation of treatment are relatively liberal, in others the fulfilment of strict criteria is required. In the UK eligibility criteria are relatively stringent, as patients must present with high disease activity (DAS28 > 5.1) after failure of intensive therapy with a combination of csDMARD (www.nice.org.uk/guidance/ta375).

There is growing body of evidence suggesting a significant benefit from early initiation of TNFα inhibitors in DMARD-naïve RA patients. Several studies have shown higher proportion of patients achieving remission and less radiographic progression in patients on MTX plus anti-TNFα compared to MTX alone. [254,257,275-279] Similarly, a sub-analysis of the BeSt study showed a better outcome of patients receiving early compared to delayed Infliximab plus MTX combination therapy. [280] A study by Quinn *et al* suggested a role for intensive induction therapy with biologics in DMARD-naïve RA, followed by maintenance with csDMARD. [281]

A number of other biologic agents aimed at targets other than TNFα are available for treatment of patients with RA: Rituximab, a chimeric human/murine monoclonal antibody targeting the CD20 antigen expressed on mature-B and pre-B cells; Tocilizumab, a humanized anti-IL6R; Abatacept, a recombinant fusion protein consisting of the extracellular domain of human CTLA4 and the Fc domain

of human IgG1, which inhibits the co-stimulatory signal required for APC dependent T cell activation. [282]

1.7.4.5 Targeted synthetic Disease-Modifying Antirheumatic Drugs

Targeted synthetic DMARD (tsDMARD) represent an emerging class of drugs for RA, that modulates a specific target implicated in the inflammatory cascade. Examples include janus kinase (JAK) inhibitors, like tofacitinib or baricitinib. Cytokines, on binding to cell-surface receptors, exert their function by inducing intracellular signalling that activate intracellular pathways. The non-receptor tyrosine kinase family JAK has an essential role in transducing cytokine-induced signals that influence normal and pathological intracellular pathways and immune cell functions, including pathogenic mechanisms involved in RA. [283]

Tofacitinib was the first approved tsDMARD for treatment of moderate to severe RA. It is an orally administered small molecule inhibitor which modulates the inflammatory process by selectively targeting the JAK3 pathway cascade. Tofacitinib is indicated for the treatment of patients with RA who have had an inadequate response to MTX and/or other DMARD. [284]

Baricitinib is a JAK1/2 inhibitor now approved for use in Europe. In phase 3 clinical trials it appears to have similar therapeutic efficacy as bDMARD and Tofacitinib in patients with moderate to severe RA. [285]

The current approach to the treatment of early RA is schematised in the algorithm shown on Figure 1.3.

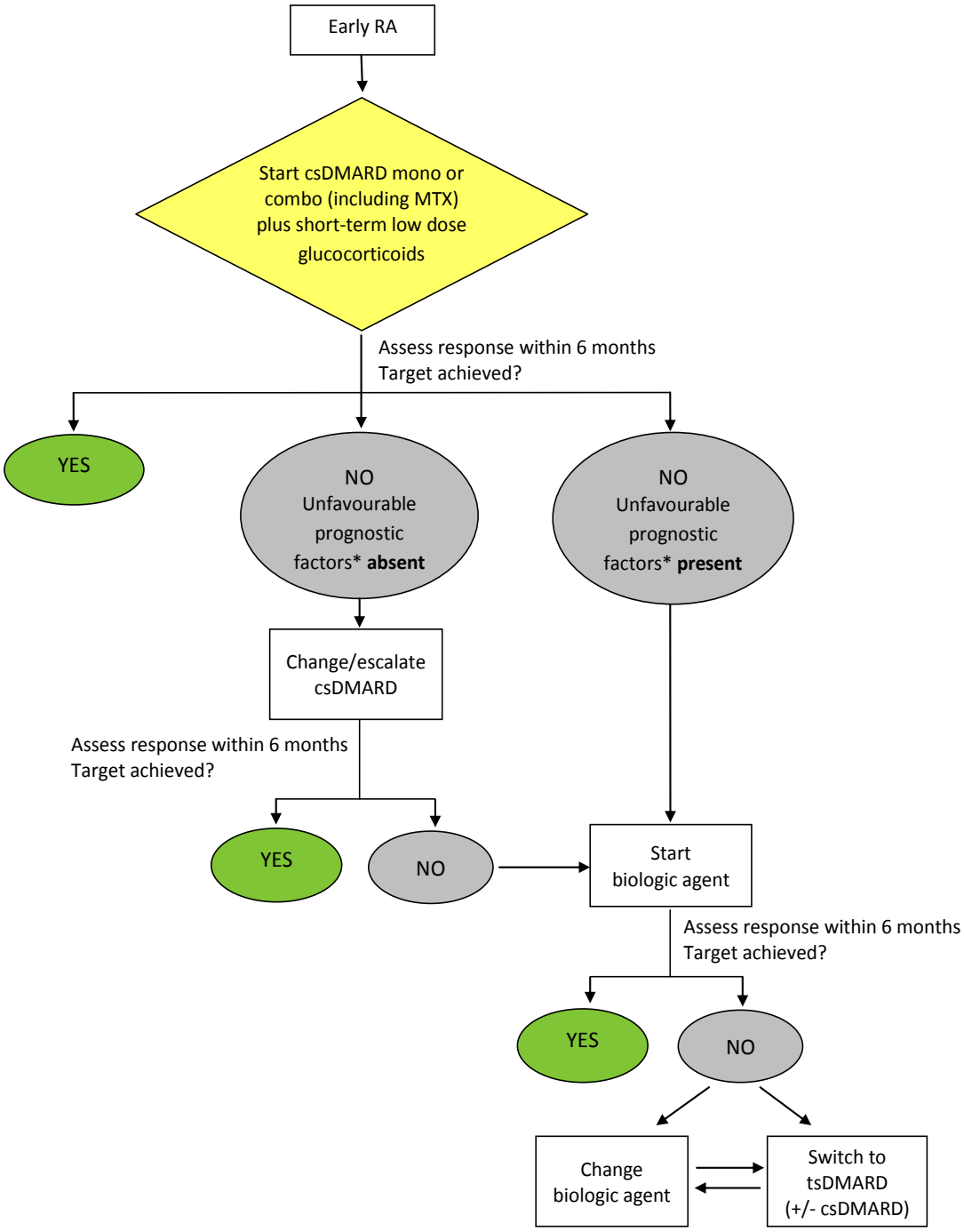


Figure 1.3: Therapeutic algorithm for the treatment of early rheumatoid arthritis
*Unfavourable prognostic factors: high disease activity, RF/ACPA positive, early joint damage.
Abbreviations: csDMARD= conventional synthetic Disease-Modifying Drugs; MTX= Methotrexate; RA= rheumatoid arthritis; tsDMARD= targeted synthetic Disease-Modifying Drugs.
Adapted from [237]

1.8 SYNOVIAL TISSUE EXTRACTION AND ANALYSIS: STATE OF THE ART

1.8.1 Recent progresses in synovial tissue acquisition

The inflamed synovium is the pathological hallmark of RA. Exploring the characteristics of the joint tissue is pivotal in enhancing our understanding of disease pathogenesis and in developing future targeted therapies. Therefore, advancement in biopsy techniques harvesting sufficient quantity and good quality tissue is essential.

A number of approaches have been attempted in order to facilitate the acquisition of tissue samples from large and small joints.

Historically, synovial biopsies have been performed by using a blind needle approach (Parker–Pearson or Williamson–Holt needle). [286] Because of the lack of direct visualization making it difficult to biopsy small joints, this technique was restricted to large joints, mainly knees. This represented a significant limitation in early arthritis studies where small joints and particularly hands and wrists are predominantly involved. [287] Large joint involvement in the early phase of the disease identifies a subset of patients with worse prognosis, therefore studies based exclusively on tissue from knees or other large joints would inevitably introduce a methodological bias. [288] Another disadvantage of this technique was the difficulty to obtain adequate tissue samples from joints that had became quiescent following effective therapy.

Due to such limitations, this methodology had been progressively replaced by arthroscopic biopsies performed under local anaesthesia, which harvest good

quantity and quality tissue under direct visualization. During arthroscopy, the operator is able to visualize the synovium macroscopically, so it is relatively easy to obtain adequate amounts of synovial tissue even when the volume of the tissue has reduced after successful treatment. To date, arthroscopy remains the gold standard for synovial tissue extraction. [289] However, arthroscopic biopsies present several issues, including technical complexity requiring specific training, high costs, pain or discomfort during the procedure reported by up to 35% of patients, [290] minor complications such as vasovagal reactions and post-procedure joint swelling observed in 5–10% of cases, more severe complications like haemarthrosis (0.9%), deep vein thrombosis (0.2%) and biopsy site infection (0.1%). [291]

More recently, advances in the use of musculoskeletal US by rheumatologists has led to the development of US-guided synovial biopsies, allowing the acquisition of synovial tissue using a minimally invasive, easy to perform and well tolerated technique. US-guided synovial biopsies offer undoubted advantages: US assessment may facilitate selection of the best joint suitable for biopsy; US images guide the operator during the procedure, facilitating site visualization with minimal invasiveness; the technique requires less intensive training and after purchase of an US machine minimal investment in equipment; it can be easily performed in a standard rheumatology setting; it allows easy approach of small joints as equally as large joints, therefore it is ideal to be utilized in early arthritis studies.

Previous studies described US-guided synovial biopsy performed using a portal and forceps technique. [292,293] This method consists in inserting a flexible wire into the joint under US guidance. The wire is subsequently removed, and a rigid

Hartmann's forceps used for the tissue extraction. This method has been validated in a small case series of nine patients with long standing RA. [292]

However in a recent survey of 93 US-guided synovial biopsies performed in the Rheumatology Department at Barts and The London Hospital, successful biopsies of large and small joints have been obtained through minimally invasive US guided technique using a 14-16G Quick-Core (Cook Medical) needle. [287] Of the 93 biopsy procedures, 57 were performed prior to csDMARD therapy and 36 were repeat biopsies after 6 months of treatment in patients recruited as part of the 'Pathobiology of Early Arthritis Cohort' (PEAC) study. Five joint sites were biopsied (knee, elbow, wrist, metacarpal phalangeal and proximal interphalangeal joints). Up to 12-15 biopsy samples from any joint site were retrieved per procedure. 86 (92.5%) biopsy procedures yielded high-quality tissue suitable for histopathological characterisation (the rate of success for renal and liver biopsies routinely performed for diagnostic purposes is 83-97% [294,295] and 81-97% [296] respectively). Notably, tissue quality was preserved in subsequent biopsies following therapeutic intervention as shown by the 36 patients who received a second synovial biopsy. This is important in the context of using such a technique to monitor changes in synovial biomarkers of response to therapy.

This data also demonstrated that the procedure is safe and well tolerated by patients. No significant complications were reported. No significant difference in the assessment of joint pain, swelling and stiffness on a visual analogue scale pre- and post-biopsy was detected (Figure 1.4). [287] Notably, biopsies of small-sized and medium-sized joints (MCPs, PIPs and wrists) were better tolerated than large sized joints (elbows and knees), and again this is relevant especially for studies aiming at

investigating pathobiology of early arthritis where involvement of small joints is predominant.

The majority of patients were agreeable to undergo a repeat procedure (Figure 1.4). This is of great importance in the context of using serial synovial biopsies to monitor the disease course and response to therapy.

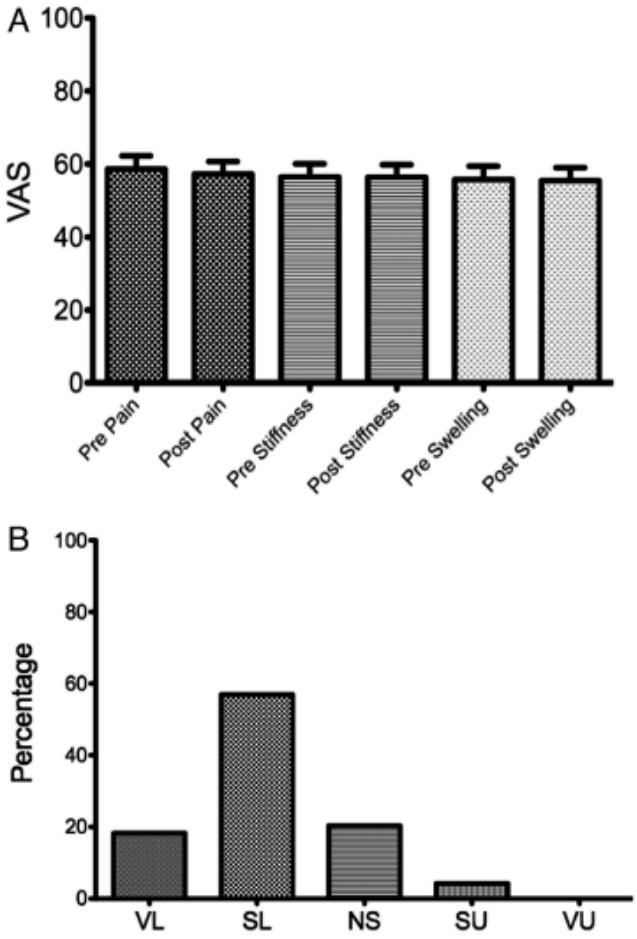


Figure 1.4 : US-guided biopsy is a safe and tolerated procedure

Patients completed a visual analogue score assessment of joint pain, stiffness and swelling prior to and after the procedure. No significant difference in any of the three parameters pre- and post-procedure was reported (A).

After the biopsy, patients were asked how agreeable there were to having a subsequent one at a later time-point: very likely, somewhat likely, not sure, somewhat unlikely, very unlikely. Results expressed as percentages of total patients (B).

Abbreviations: NS= not sure; SL= somewhat likely; SU= somewhat unlikely; VAS= visual analogue scale; VL= very likely; VU= very unlikely.

From [287], with permission.

1.8.2 Methodological issues related to synovial tissue extraction and analysis

In order to make synovial pathobiological analysis a reliable and reproducible tool, a number of critical issues need to be addressed, and specifically: i. is a single joint truly representative of the other inflamed joints? ii. does a single joint area faithfully reflect the inflammation in the whole joint?, iii. how many biopsy specimens from the same joint are necessary to be analysed to ensure an accurate representation of the whole joint?, iv. how many sections from a single biopsy ? and , v. what is the minimum tissue area/sample size required for analysis? [297]

To address whether the cellular infiltrate of a single joint is representative of other inflamed joints, a study from Kraan and colleagues compared the characteristics of the cellular infiltrate in paired biopsies of large joints (knees) and small joints (wrists or MCPs) of nine patients with RA, showing no significant differences in any of the cellular and molecular biomarkers. [298] This provides proof of concept that the cellular infiltration of a single joint is truly representative of the systemic disease process. However potential limitations of the study were the small sample size and the fact that patients were at different disease time-points ranging from early to long-standing disease and had heterogeneous treatment exposure.

Another crucial issue is to establish the minimum amount of tissue required for analysis. Based on multiple analysis of variance, it was established that a minimum of six to eight tissue samples is required in order to avoid overestimation or underestimation of the inflammatory infiltrate and provide a reliable representation of cellular/molecular components of the whole joint. [289] This observation was originally made focusing on synovial tissue obtained surgically

from knee joints from 3 patients with RA [299]. Another study on US-guided biopsies of hand joints of nine patients with RA, demonstrated that the analysis of a 2.5 mm^2 tissue section provides a reliable estimate of synovial macrophages (CD68-positive cells), T cells (CD3-positive cells) and B cells (CD20-positive cells) count, within 10% variability of the total sample mean. [292]

Recently published data aiming to determine the degree of variation of synovial cellular infiltration of CD3, CD20 and CD68 between single joint biopsies obtained from different patients as well as between and within biopsy specimens from the same joint, in order to standardize the minimum quantity of synovial tissue necessary to obtain a representative picture of the immunohistochemical environment by using US-guided needle synovial biopsy of small joints.[300] The results showed a significant variation in the degree of cellular infiltration between patients and between biopsy specimens extracted from the same patient, but no significant variation within multiple sections from the same biopsy specimen. The authors concluded that, rather than examination of multiple sections from the same specimen, analysis of multiple biopsy specimens is required. They demonstrated that the examination of a minimum of 4 biopsy specimens gives an accurate representative assessment of CD3, CD20 and CD68 cellular infiltrate. Finally, they aimed to determine whether pre-biopsy US assessment of synovitis was useful to predict synovial tissue yields, reporting that joints with greater synovial thickening yielded better tissue in terms of both quantity and quality, meanwhile the presence of Doppler signal did not predict the success of the procedure. These observations remark that US assessment represents a powerful tool for joint site selection and maximization of tissue extraction.

1.8.3 The characteristics of the normal synovium

To investigate the synovial tissue pathobiology, an essential prerequisite is to understand the microarchitecture of the normal, uninflamed synovium.

Synovial tissue lines the surface of diarthrodial joints, tendon sheaths and bursae. It consists of two anatomically and functionally distinct compartments: an intimal layer of 1-2 cells (intima) which is in contact with the articular cavity, and an underlying tissue (subintima) containing blood vessels, fat cells, resident fibroblasts and a small number of infiltrating cells (macrophages, lymphocytes and plasma cells) surrounded by collagenous extracellular matrix.

The physiological functions of synovium are various, including to confer a non-adherent surface with viscoelastic properties allowing joint movement and stretching, to provide lubrication of cartilage, to regulate the composition and the volume of the synovial fluid and to ensure the nutrition of the articular chondrocytes. [80,301]

Two types of cells compose the intimal layer: type A synoviocytes, deriving from the macrophage lineage (macrophage-like synoviocytes), and type B synoviocytes, deriving from the fibroblast lineage (fibroblast-like synoviocytes, FLS). Type A synoviocytes are true macrophages derived from hematopoietic cells and migrating into the synovium from the bone marrow via circulation, however it is not clear whether their differentiation occurs in the bone marrow or in situ. The intimal macrophages strongly express CD163 and CD68 but less CD14, while subintimal macrophages mostly express CD14. The number of intimal macrophages is very scarce in normal conditions, meanwhile they may represent

the predominant cell population in patients with inflammatory arthritides, accounting up to 80% of total number of cells. [301]

Type B synoviocytes are mesenchymal cells displaying classic characteristics of fibroblasts including expression of type IV and V collagen, vimentin, vascular cell adhesion molecule-1 (VCAM-1), CD90 and CD55. It is not clear whether they migrate from the subintima or are a locally derived population. They secrete hyaluronic acid and lubricin, which are critical proteins for lubrication of cartilage. [301] Type B synoviocytes have some peculiar properties that distinguish them from other fibroblast lineages, including sublining resident fibroblasts. They specifically express cadherin-11, an adhesion molecule that plays a key role in cell-cell adhesion in a variety of cell types and is up regulated in several forms of cancer and other pathological conditions including inflammatory arthritis. Mice models that are cadherin-11 deficient present a less structurally defined synovial intimal lining and are refractory to the development of joint inflammation.

1.8.4 The characteristics of the rheumatoid synovium

There are no clear-cut histological features that differentiate RA from other forms of chronic inflammatory arthritis. [302] Studies aiming at comparing the characteristics of RA with psoriatic arthritis (PsA) synovium showed that, although some aspects may be more typical of PsA -less pronounced intimal lining thickening, more pronounced vascularity, prevalence of innate over humoral response- they show more similarities than differences. [303-305] However such results should be interpreted with caution, as these works were performed on

small groups of patients with longstanding disease and exposure to previous treatment.

Macroscopically, RA synovium is characterised by hypertrophic and oedematous tissue with prominent villi extending into the articular space, along with profuse synovial fluid effusion. Microscopic analysis shows that the critical component of the inflammatory process since early stage are cellular infiltration, neoangiogenesis and hypervascularity. RA synovium is in fact characterised by three major histopathological alterations: thickness of the intimal layer due to infiltration of monocytes/macrophages and proliferation of FLS; heavily infiltrated subintimal region by a diverse array of inflammatory cells -predominantly macrophages and CD4/CD8 T lymphocytes, but also B lymphocytes, plasma cells, neutrophils, NK, mast cells and DC- and proliferation of resident synovial fibroblasts; marked neoangiogenesis in the context of synovial tissue hypoxia as the result of the production of pro-angiogenetic factors such as VEGF and hypoxia-inducible factors (HIFs). [302,306,307]

According to the distribution of the immune cells within the synovial sublining, the synovial infiltrate has been long distinct into two mutually exclusive patterns: a diffusely distributed infiltrate, where lymphocytes are intermixed with macrophages and resident fibroblasts (‘diffuse’ pattern, Figure 1.5), and a highly organised infiltrate where T and B cells cluster into lymphocytic aggregates (‘aggregate’ pattern, Figure 1.6). [308] In up to 25% of patient these structures can acquire a high degree of organization including the compartmentalization of T and B cells in discrete domains, the differentiation of high endothelial venules (HEV), the localisation of mature DC, and the acquisition of FDC networks, leading to the

formation of germinal centres (GC) with local immunoglobulin gene somatic mutations. These highly organised structures resemble secondary lymphoid organs and may acquire the functionality of ectopic lymphoid neogenesis (ELN).

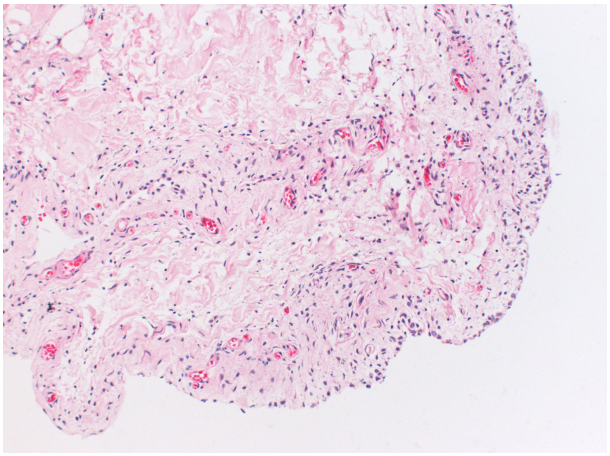


Figure 1.5 : Inflamed synovium characterised by 'diffuse' synovitis
Cellularity is slightly increased with diffuse and perivascular inflammatory infiltrate visible in the stroma.
Picture from the Pathobiology of Early arthritis Biobank (PEAC) biobank. Courtesy of Prof. C. Pitzalis.

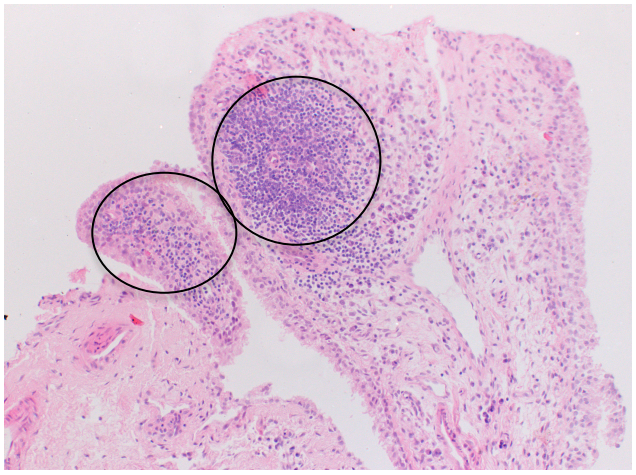


Figure 1.6 : Inflamed synovium characterised by 'aggregate' synovitis
Cellularity is moderately increased with two large follicle-like lymphocytic aggregates (black circles).
Picture from the Pathobiology of Early arthritis Biobank (PEAC) biobank. Courtesy of Prof. C. Pitzalis.

The immunophenotypic characterisation of these two main histo-morphological patterns demonstrates B cells principally in aggregate synovitis (30–40% of patients) and CD68+ macrophages principally in diffuse synovitis. However recent reports have shown that the synovial infiltrate has a more continuous spectrum than the mutually exclusive distinction between these two patterns.

Recently, a third pattern has been described, Pauci-immune (PI), that shows hardly any classic infiltrating immune-cells and yet is found in active disease. [297] This has led to a new histological classification based on immunophenotypic and cellular characterisation rather then on mere architectural organisation: Lymphoid (lymphocytes-dominant with presence of synovial aggregates), Myeloid (macrophages-dominant with absence of aggregates) and PI (low/absent infiltrate and predominance of stromal tissue). A representative example illustrative of RA synovial membrane with these three histo-morphological appearances is shown on Figure 1.7.

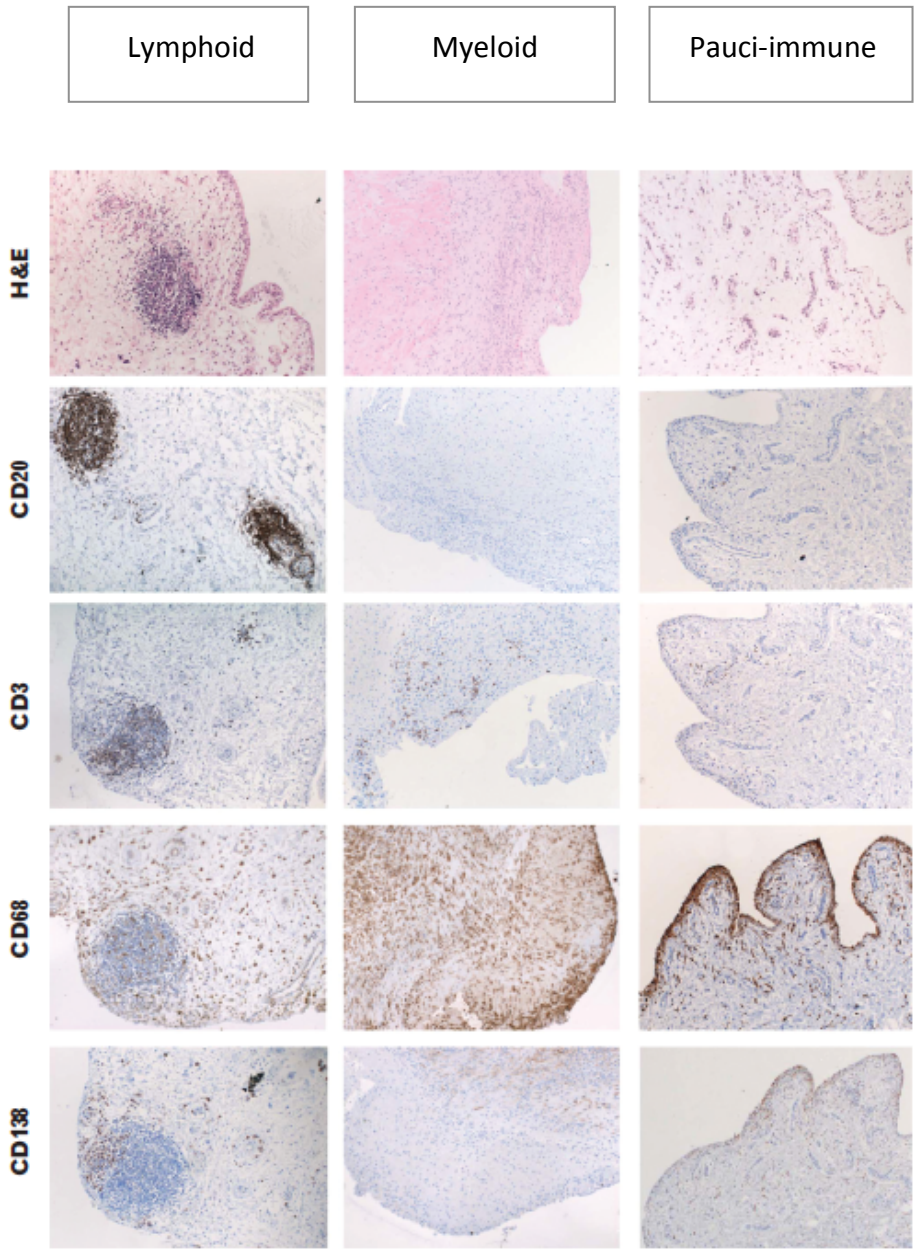


Figure 1.7 : Three distinct histomorphological patterns of rheumatoid synovitis
Microphotographs of prototypical examples of Lymphoid, Myeloid and Pauci-immune synovitis. Three mm sections of paraffin embedded RA synovial tissues were stained with Haematoxylin and Eosin (H&E) and by Immunohistochemisty for B cells (CD20), T cells (CD3), Macrophages (CD68) and Plasma cells (CD138).
From [297], with permission.

Notably, these histo-morphological patterns segregate with specific transcriptomic signatures: the analysis of a cross-sectional cohort of samples from 49 RA patients, revealed transcriptomic clustering into Lymphoid, Myeloid and two Fibroblast (F,F/A) prevalent patterns, together with a mixed pattern. [297] The presence of these two fibroblasts patterns is in line with previous reports showing the coexistence in the RA synovium of FLS showing a transforming-growth-factor-b /activin-A inducible signature characteristic of myofibroblasts, and FLS displaying principally an insulin-like-growth-factor regulated genes signature. [309] The transition from normal FLS to myofibroblasts-like FLS is associated with the expression of matrix degrading proteases [310] that might explain the aggressive phenotype acquired by these cells in RA patients [311].

The fact that the PI pattern - not constituted by classic immune cell infiltrate and still associated with active disease - has been genetically identified with an abundant fibroblastic expression is of great interest given the emerging evidence of a key role of these cells in the pathogenesis of RA. [76]

Confirming the above findings, in a recent publication Dennis *et al* described 3 main patterns emerging from genome-wide analysis of synovial tissue: Lymphoid (B cells and plasma cells dominated), Myeloid (macrophage dominated), Fibroid (hyperplastic, pauci-immune tissue) along with an overlapping 'Low-inflammatory' pattern, indicating that synovial heterogeneity exists as a biological continuum rather than fixed, mutually exclusively patterns. Notably, histological and flow cytometry analysis were consistent with the dominant gene-expression defined inflammatory subsets: the Lymphoid pattern was associated with the presence of

synovial aggregates, the Myeloid characterised by a diffuse immune infiltration and the Fibroid showed little infiltration and complete absence of aggregates. [312]

As the presence of ELS is observed only in a proportion of patients, a critical issue is whether lymphoid aggregate formation represents either a fixed pattern or the transient phase of a dynamic process potentially involving all patients during the course of the disease. For example, one could speculate that the PI and the Myeloid synovitis are simply the precursors of higher grade of tissue organisation that may evolve toward the formation of lymphocytic aggregates. Data emerging from the literature is conflicting. Weyond and colleagues observed, from the analysis of several biopsy series collected years apart, that the synovial pattern was consistent for each patient and remained stable over time. [313] Conversely, a recent study by van de Sande based on the analysis of 17 repeated biopsies six months apart, showed that the presence of lymphocytic aggregates is a dynamic phenomenon that may vary over time. [314] Methodological discrepancies, including the non-homogeneous definitions of aggregates utilized across the above studies, the confounding role of therapeutic interventions, the inclusion of patients at different stages of disease may account for these conflicting conclusions, highlighting that the lack of methodological uniformity is the main caveat in this field of research.

1.8.5 Synovial ectopic lymphoid structures: immunological and clinical aspects

As previously mentioned, T and B lymphocytes may be found randomly sparse within the synovium or clustered to form discrete perivascular aggregates (ectopic lymphocytic aggregates, ELS) establishing close connections with macrophages,

synovial fibroblasts and DC. These aggregates can ultimately acquire the specific morphological and functional characteristics of ectopic lymphoid structures. [315,316]

The presence of ELS is an intrinsic feature of chronic inflammation and has been described in a large spectrum of autoimmune diseases other than RA, including Sjogren's syndrome, thyroiditis, myasthenia gravis, multiple sclerosis. Furthermore, they are not restricted to autoimmunity but can be found in other forms of chronic inflammation, fibrosis, granulomatosis, chronic infections (e.g. hepatitis, gastritis, protozoa-induced diseases) and cancer, indicating that this type of immune response is not disease or organ specific and is not representative of a unique pathological process. [317]

The genesis of ectopic lymphoid tissue is not fully understood. The main hypothesis is that a non efficient removal of the antigenic stimuli -derived either from an extrinsic on an intrinsic source- may result into a persistent antigenic stimulation leading to the up-regulation of specific chemokines and adhesion molecules patterns, promoting the dysregulation of T cells- B cells- dendritic cells interactions and determining the persistence of a chronically inflamed environment. [318]

Several molecular pathways contribute to the formation of ectopic lymphoid tissue. These include the aberrant in situ production of lymphoid chemokines (CXCL13, CCL19 and CCL21) and cytokines (LTα and LT β) that are constitutively involved in the homeostatic maintenance of lymphoid tissue. [313,319] Lymphoid chemokines are instrumental in regulating trafficking of naive and memory T–B lymphocytes within the ectopic lymphoid tissue. The molecular remodelling acquired by the vasculature of the inflamed synovium, with the acquisition of HEV

and the luminal expression of peripheral node addressin (PNAd) to which lymphocyte expressing L-selectine can bind, is also critical to promote adhesion and internal transmigration of lymphocytes. [320]

A close relationship exists between the degree of aggregation and the acquisition of specific lymphoid tissue features. Indeed the size of lymphocyte aggregates appears to translate to function, with bigger sizes corresponding to higher lever of organisation and function. Histomorphometric analysis based on the aggregate radial cell count has led to the development of a validated grading system based on a 1-3 scale dimensional grading: the grading is established according to the radial lymphocyte count from the central blood vessel to the point of highest cell infiltration within the selected tissue area, with grade 1 (G1= two to five cells) defining small-size, grade 2 (G2= six to ten cells) and grade 3 (G3> 10 cells) defining large-size lymphocytic aggregates. [319,321] The acquisition of structural and functional features constitutively of secondary lymphoid tissue organisation is observed in large aggregates only. [322] The presence of large-size aggregates is seen in approximately 30%-40% of RA patients. [323,324]

Much debate has emerged about the pathophysiological significance of ELS in RA. The main question is whether they represent only a bystander of chronic inflammation, or are truly implicated in disease pathogenesis. Indeed, evidence is accumulating that ELS are not just an epiphenomenon of local inflammation but play a key role in activating and perpetuating the immunological response in RA.

A direct functional role in inflammation and autoantibody production for synovial ELS has come from a number of studies including one using the human RA SCID (Severe Combined Immunodeficiency) mouse model.[325] This study

demonstrated that synovial graft ELS support the proliferation and differentiation of B cells as well as the expression of Activation-Induced Cytidine Deaminase (AID), an enzyme which is critical for class switch recombination and affinity maturation of antibodies, and this correlated with human ACPA titres (IgG) within serum (Figure 1.8). These events occurred in the absence of new immune cells infiltrating the grafts (SCID are in fact immune deficient) indicating that ELS contribute to disease pathogenesis via self-sustained immune activation.

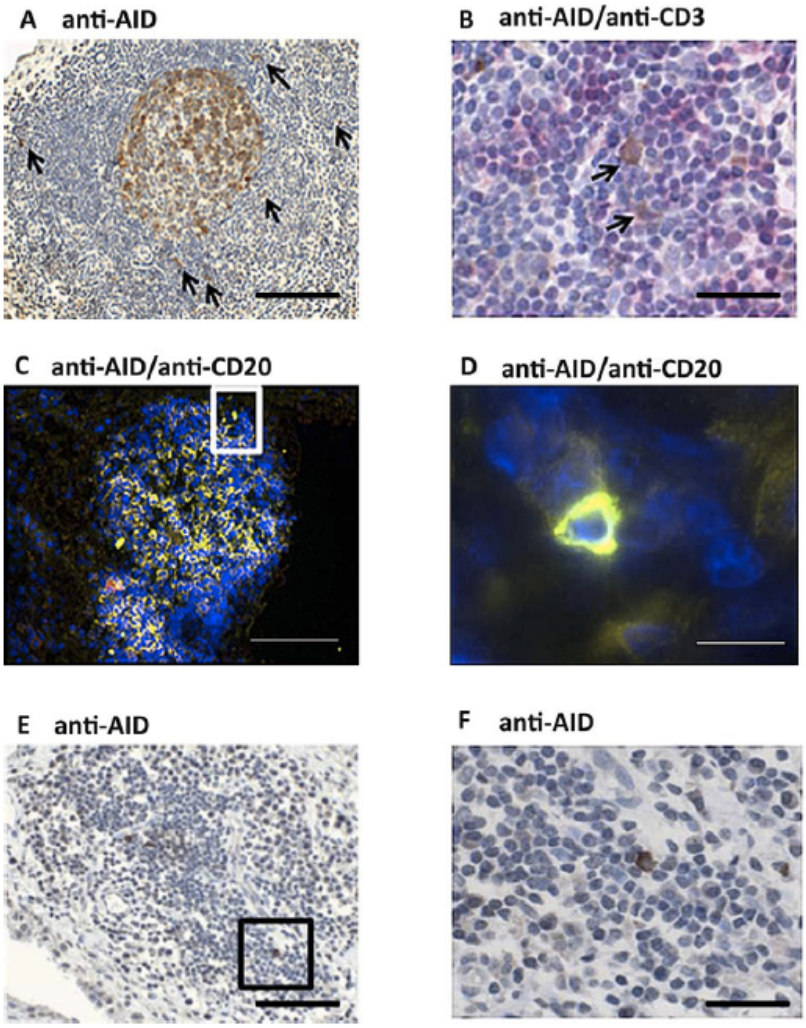


Figure 1.8 : Expression of Activation-Induced Cytidine Deaminase (AID) enzyme, which is critical for the processes of class switch recombination and affinity maturation of antibodies, identifies large B cells aggregates within the rheumatoid synovium

(A) Paraffin-embedded sections from RA patients were stained for AID (203magnification). AID+ cells (arrows) were frequently and exclusively seen in RA synovial tissue characterised by the presence of large aggregates.

(B) Paraffin sections were double stained for AID (brown) and CD3 (red) (603magnification), demonstrating the close relationship between AID+ cells (arrows) and T cells in the peripheral T cell areas of the lymphoid aggregates.

(C) Merged double staining for AID (red) and CD20 (green) on frozen RA sections confirmed that AID+ cells were of B cell origin (double-stained cells are identified in yellow).

(D) Higher magnification of an example of an AID/CD20 cell (603 magnification of [C]).

(E and F) Scattered AID cells (E) with the appearance of interfollicular B cells (F) were occasionally found away from the central focus of the aggregate.

Scale bars: 200 lm (A, C, E), 50 lm (B, F), 15 lm (D).

Abbreviations: AID= Activation-Induced Cytidine Deaminase enzyme; CD3= T cells, CD20= B cells; RA= rheumatoid arthritis.

From [325], with permission.

Nonetheless, lymphoid aggregates are also found in seronegative types of arthritis such as spondyloarthritis and PsA. Canete *et al* analysed 27 knee arthroscopic samples from PsA patients specifically looking for T/B cell aggregates and expression of chemokines associated to lymphoid neogenesis like CXCL13 and CCL21: they found that up to 25 of 27 synovia contained aggregates, of which up to 60% were large size aggregates. [326]

The presence of lymphoid aggregates in the synovium has been associated with higher levels of pro-inflammatory mediators involved in the pathogenesis of RA, supporting a direct functional role for autoantibody and pro-inflammatory mediators production by such structures. Yanni *et al* reported significantly higher levels of IL-1, IL-6, and IL-2 in synovial tissue with lymphoid aggregates when compared to tissue with a diffuse infiltrate, suggesting that the presence of ectopic lymphoid tissue could reflect a more aggressive disease process. [327] In line with these findings, Klimiuk *et al* demonstrated the existence of a unique cytokine profile in different variants of synovitis, with typically lower expression of IFNγ, IL-4, IL-1 and TNFα in diffuse synovitis. [308] Thurlings *et al* also observed a higher expression of TNFα producing cells in the sublining of aggregates-reach synovium, along with increased levels of serum biomarkers of systemic inflammation. [323]

Finally, a direct contribution of synovial lymphoid aggregates to radiographic progression has been hypothesised. Kotake et al detected higher expression of RANKL from activated T cells within synovial aggregates, suggesting a possible direct role in the induction of local osteoclastogenesis and bone destruction. [328] In keeping with these observations, Klimiuk *et al* reported a more advanced radiographic status in patients with synovial aggregates compared to patient with

no aggregates by analysing end-stage joint replacement tissue samples. [329] These findings were not confirmed by two subsequent studies. [323,330] Most of these observations derive from studies conducted in heterogeneous cohorts of long-standing/end-stage disease, with a significant number of confounding factors.

1.8.6 Sensitivity to change of the synovium after therapeutic intervention

Finally, an important question is whether effective treatment of RA is able to induce changes in the synovium in parallel with changes observed clinically.

The evaluation of serial synovial biopsies has demonstrated that successful treatment of RA is effectively associated with ability to modulate synovial inflammation, as assessed by decrease of cellular infiltrate and expression of cytokines, chemokines and adhesion molecules. [331] In particular, a number of studies have suggested that clinical response to treatment correlates with a significant decrease in the number of synovial sublining macrophages (CD68sl). As such CD68sl has been recommended as a valid biomarker of response to treatment in clinical trials. A study from Haringman and colleagues collecting 88 patients with established RA, showed that the mean change in disease activity correlated well with the mean change in the number of CD68sl, and this was independent of the therapeutic strategy. [64] This observation was consistent across centres [332], suggesting a realistic potential for synovial tissue analysis as a tool to evaluate efficacy of anti-rheumatic treatment. There is however limited data examining synovial CD68sl response in treatment naïve early arthritis patients.

Given the growing evidence that ELS are functional and potentially involved in disease pathogenesis, much interest is focused on the potential ability of effective

treatment to disrupt/revert these structures. A study from Canete and colleagues looked at 24 paired synovial biopsies performed prior and after TNFα inhibitors: of the 16 patients who presented with lymphoid neogenesis in the first biopsy, 7 remained stable meanwhile 9 turned into a lymphoid neogenesis negative status, and this change correlated with clinical response. [330] Similarly, Klaasen and colleagues observed a reduction in the number and size of lymphocyte aggregates following effective anti-TNFα therapy, although this did not reach statistic significance possibly due to the small sample size. [324]

It has been observed that treatment with anti-CD20 depleting agents such as Rituximab induces not only a decrease in synovial B-cell density, but also a disruption of the lymphoid architecture and a reduction of cytokine expression, T cells, plasma cells, FCD and subintimal macrophages. These observations support a direct homeostatic role for B cells in induction and maintenance of synovial aggregates, and provide evidence that B lymphocytes play an active role in orchestrating the synovial inflammatory network in situ. [333,334]

The ability to modulate the inflamed synovium does not seem to be drug-specific but has been observed in several studies comparing pre- and post-treatment biopsy series following different therapeutic strategies including Rituximab [335], Tocilizumab[336] and Abatacept[337]. Conversely, the analysis of serial synovial samples from RA patients who received either placebo or ineffective therapy did not reveal any significant change. [338]

1.9 FROM DIAGNOSTIC TO PROGNOSTIC CATEGORIES: RE-THINKING THE DIAGNOSIS AND THE CLASSIFICATION OF RHEUMATOID ARTHRITIS

At the time of first presentation to the EAC, about 30% of patients with inflammatory arthritis cannot be categorized into a definite diagnostic group and are labelled as undifferentiated arthritis (UA). [201]

From the analysis of early arthritis registers, including the Leiden Early Arthritis Clinic in the Netherlands and the Norfolk Arthritis Register in the UK, it has emerged that up to 40-50% of UA patients have a self-limiting course that usually resolves within a few weeks or months; about one third will evolve toward persistent inflammatory arthritis including RA or PsA; and the remaining will stay undifferentiated or develop other rheumatologic conditions. [339-341]

Unfortunately, the benign/self-limiting forms of UA cannot easily be distinguished from the ones that will progress toward chronic/erosive arthritis just as those patients originally identified as having RA. This means that some patients with an early inflammatory arthritis may not be identified and treated promptly; the opposite scenario would be over-estimating mild, self remitting forms of arthritis, resulting in over-treatment and exposure to risk of drug toxicity unnecessarily.

It is thus important to determine clinical, serological and histopathological markers of persistent disease and joint damage to allow the identification of those patients in need of prompt aggressive treatment. Future challenge in rheumatology would be the stratification of patients with early inflammatory

arthritis according to severity/prognostic categories rather than diagnostic/classification criteria, in order to tailor treatment strategies at the individual level.

1.9.1 Prediction models for the diagnosis of rheumatoid arthritis

Currently, the strongest independent predictor of disease progression in early inflammatory arthritis is the presence of ACPA antibodies. [99] However, there is no single predictor showing sufficient discriminative power, with data demonstrating that the combination of more than one factor is the key to improve prognostic value.

The first prediction model for RA was originally proposed by Visser and colleagues in 2002. [95] This model was derived from a cohort of 524 patients who attended the Leiden Early Arthritis Clinic, and consisted of seven variables: symptoms duration, morning stiffness for at least one hour, arthritis in 3 or more joints, bilateral compression pain in the MTP joints, seropositivity for RF, seropositivity for ACPA, and presence of erosions in hands or feet. It showed excellent ability to discriminate between self- limiting, persistent non-erosive and persistent erosive arthritis at two years follow-up: the ROC area under curve for discrimination between self-limiting and persistent arthritis was 0.84 and for discrimination between erosive and non-erosive arthritis was 0.91. In comparison, the discriminative power of the 1987 ACR classification criteria was significantly lower. Notably, ACPA independently and significantly contributed to the performance of the model. [95] Two major limitations of this work have been recognised. First, not all patients were treatment naïve at the time of recruitment,

as many had received or were receiving MTX or other csDMARD. The decision to start csDMARD therapy could have been influenced by the same clinical variables that were included in the model, leading to circularity. Second, the definition of early arthritis included disease duration up to 2 years, a time frame that cannot longer be accepted in light of the recent concept of window of opportunity.

A subsequent prediction rule was developed by van der helm-van Mil's and colleagues, [96] which was again derived from the Leiden cohort in the Netherlands. This model aimed to determine the likelihood of a patient with early inflammatory arthritis fulfilling 1987 criteria for RA within the first year from symptoms onset. The tool consists of nine variables: age, gender, distribution of involved joints, severity of morning stiffness, number of tender joints, number of swollen joints, CRP, seropositivity for RF and ACPA. In the derivation cohort, patients with ≤6 points had an absolute chance of not developing RA of 91%, meanwhile patients with ≥8 points had a 84% chance of progressing toward RA. Some criticism related to methodological aspects of this model has been raised too. Firstly, approximately 20% of patients' scores fall between the cut-off values of 6 and 8, so they cannot be classified. Secondly, a disadvantage of using positive predictive value (PPV) and negative predictive values (NPV) is that these measures are dependent on the disease prevalence that may vary across populations -the higher the incidence of RA, the higher the PPV and the lower the NPV. Nonetheless, the diagnostic accuracy of the prediction rule was preserved in validation studies performed across different cohorts. [340] Finally, as 3 of the 9 criteria that form the prognostic algorithm are also part of the ACR 1987 criteria, Visser disputed the choice of fulfilment of ACR 1987 criteria as the outcome measure at one year

suggesting this may have led to incorporation bias and circularity. He stated that using a clinical outcome (such us persistence of arthritis) rather than fulfilment of set criteria could have been more appropriate. [342] The authors replied that, as there is not clear-cut definition for 'persistence of disease', they preferred to avoid such an indefinite outcome measure and rather use a validated one.

Since synovial inflammation is the pathological hallmark of arthritis, some studies have aimed at exploring if the characteristics of the synovial tissue may be able to facilitate a clinical diagnosis of RA in patients with new onset of arthritic symptoms. van de Sande *et al* analysed 69 arthroscopic biopsy samples that revealed no clear-cut differences in the features of the synovium between RA patients initially diagnosed as UA and subsequently re-classified as RA and those who originally fulfilled classification criteria for RA. [343] A further study analysing arthroscopic biopsy samples from 93 early arthritis csDMARD naïve patients, revealed no relationship between the presence of aggregates at baseline and the fulfilment of a definitive diagnosis after 2 years follow-up. In particular, the presence of large aggregates at baseline was observed in about one-third of all diagnostic subgroups, with no clear-cut differences between patients with self-limiting disease and those who developed persistent disease. [314] Conversely, an analysis of synovial biopsies from 95 patients with early inflammatory arthritis conducted by Kraan *et al* revealed that higher scores for plasma cells, macrophages and CD22+ B cells were sensitive markers to differentiate RA from other diagnostic categories. [344]

1.9.2 Prediction models for the prognosis of rheumatoid arthritis

The clinical spectrum of RA ranges from a mild, non-erosive to an aggressive phenotype resulting in rapid joint destruction. Indeed, rather than a single disease, RA could be defined as a clinical syndrome encompassing several disease subsets.

Predicting the outcome of RA at a group or even individual level would be most relevant for therapeutic decision-making, and especially identifying those patients at risk of radiographic progression and poor response to treatment. [345]

A number of factors including presence of RF and ACPA, disease duration, impaired functional status, presence of radiographic erosions, smoking status and high inflammatory markers are associated with a more severe clinical course and worst outcome. ACPA, in particular, have recently consolidated their role as strong predictors of radiographic progression. [207,346-350] However, the prediction accuracy of each individual factor is low and a number of prediction models embracing multiple parameters have been developed, with data demonstrating that combining factors improves predictive power. For example, a model incorporating anti-CCP, IgM RF, female gender and ESR, was reported to have an accuracy of 73.6% to predict radiographic progression, with ACPA representing the strongest contributor to the prediction rule. In addition, this work showed that the titre of ACPA adds prognostic information, as patients with higher level of antibodies were more prone to develop erosions compared to patients with low or moderate levels. [351] A recent report has shown that two preliminary visual matrix models including 28 swollen joint count, RF seropositivity and CRP or ESR were able to identify those patients at risk of rapid radiological progression. [352]

Several studies have looked specifically at prognostic markers of erosive disease in early RA, providing cumulative evidence for the role of autoantibodies, and particularly ACPA, as predictors of radiographic changes in the long-term. [353-356] A study from Sanmarti et al showed that the presence of ACPA was the most important contributor to a multivariate model for radiological progression in a cohort of early RA patients treated with csDMARD and low doses of steroids. [357] Machold and colleagues observed that, in an inception cohort of 55 patients with very early RA (≤3 months onset), erosions appeared over 3 years in 63% of patients despite early treatment, with a vast majority already within the first year (74%) and seropositivity for either RF or ACPA contributed to explain radiographic progression. [358]

1.9.3 Contribute of imaging to a prognostic model for rheumatoid arthritis

The presence of erosions on plain radiographs of hands and feet at baseline is a strong predictor of further deterioration. [347] However this is not particularly helpful in early RA, as the presence of erosive changes indicates failure to identify patients during the window of opportunity period, when the prevention of structural damage should be a realistic target. Therefore, efforts have been made to focus on imaging techniques that are able to provide valuable diagnostic and prognostic information at early stage. MRI and US have both shown superiority compared to plain XR in the early diagnosis of RA and monitoring of its progression.

MRI has demonstrated to be able to identify bone erosions [359] as well as synovitis and bone marrow oedema at very early stages. [220,360] A study conducted

by Navalho *et al*, showed that MRI-detected synovitis of carpal joints and flexor tendons was the most powerful predictor of progression toward RA in a population with recent-onset polyarthritis. [361] Machado *et al* reported that MRI bone oedema along with combined MRI synovitis and erosive pattern was helpful to predict progression of UA toward persistent RA, meanwhile the absence of MRI synovitis was useful in excluding development of RA. [362] Duer-Jensen *et al* confirmed these findings. [363] Hetland *et al* reported that MRI bone oedema was the strongest predictor of subsequent radiographic progression in early RA. [364]

Compared to MRI, US has the advantage of being cheaper, reproducible, with virtually no contraindication and, of most importance, can be performed and interpreted by rheumatologists in a standard clinic setting, allowing real time clinical decisions guidance.

Ultrasonography has proved to be a powerful tool to estimate the presence and the extent of inflammation within the synovium [365,366] US imaging of synovial joints provides an objective assessment of synovial hypertrophy -referred to as synovial thickening ultrasound (STUS), and vascular flow within the synovium -referred to as power doppler ultrasound (PDUS). PDUS, in particular, has attracted increasing attention in research and clinical practice, as it has been demonstrated to correlate well with inflammatory markers, disease activity and outcome. [367] Furthermore it is acknowledged that neo-angiogenesis and hypervascularity represent early, critical aspects to synovial inflammation and structural damage. [368] Neo-angiogenesis is defined as the development of new blood vessels from the existing microvascular bed. This process is mediated by pro-angiogenic factors

such as VEGF and pro-inflammatory cytokines, such as TNFα, IL-1 and IL-6, which have a direct influence on neo-angiogenesis via VEGF-dependent pathways.

PDUS shows important prognostic implications. Naredo *et al* reported that PDUS-detected synovitis in early RA is predictive of disease activity and radiological progression. [367] A milestone study by Brown *at al* showed that the persistence of PDUS signal in asymptomatic MCP joints was predictive of 12 fold higher risk of future joint damage in RA patients who were in clinical remission. [226] Other studies have demonstrated the ability of persistent PDUS signal to predict short-term relapse in patients who fulfilled clinical remission criteria [369], reinforcing the concept of imaging remission beyond the classic notion of clinical remission. Notably, Seymour *at al* reported that US of the MCP joints is a sensitive and reliable indicator of response to therapy in early RA. [370]

The incorporation of US within current prognostic models seems to provide additional predictive value. Freeston *et al* demonstrated the diagnostic benefit of adding ultrasonographic examination of the MCP joints, wrists and flexor tendons to conventional clinical tools (antibody status, ESR/CRP, radiographic damage) for the diagnosis of very early inflammatory arthritis. The predictive algorithm appeared particularly helpful in seronegative patients, with a probability of diagnosis increasing from 30% to 94%. [371] A recent study by Filer *et al* on 58 patients with very early arthritis (< 3 months), showed that the incorporation of grey scale and power Doppler assessment of the MCP, wrist and MTP joints into the van der Helm prediction rule resulted in an increase of the predictive value of the model. [372]

However several issues regarding the methodology of US assessment remain to be addressed, such as the scoring method to be utilized and the minimum number of joints to be included in the US examination. In theory, including the highest number of joints should provide the most faithful picture of the overall synovitis; however, if multiple joints need to be scanned repeatedly, the procedure would result time consuming and unfeasible for use in daily practice. Therefore, a reduced number of target joints should be ideally selected, or at least the minimum possible number of joints allowing to get exhaustive information and at the same time making the US assessment a practical tool in real life settings. Despite significant efforts undertaken over the past few years at the OMERACT level, a final validation has not been achieved yet. [373] Naredo *et al* proposed a 4-point semi-quantitative PDUS imaging of 28 joints, named the 'overall US joint index for power Doppler signal'. [367] Subsequently the same group proposed a simplified 12 joint set (elbows, wrists, 2nd and 3rd MCP joints, knees and ankles) showing an excellent correlation with a more extended 60-joint score as well as with clinical and laboratory parameters. [374] Backhaus *et al* developed a composite US score evaluating 7 joint regions (wrists, 2nd and 3rd MCP and PIP joints, 2nd and 5th MTP joints) named the 'German US7 score'. [375] In another study Seymour *et al* have shown the reproducibility and the capability to reflect synovial inflammation and detect short-term post-treatment changes of a 10 joint set including 1st-5th MCPs bilaterally. [370] Overlooking the literature, the number of joints assessed ranged from 5 to 44 across studies, with the wrist and MCP joints of the dominant hand scanned as a minimum set. [227,376]

1.9.4 The synovial biopsy as a potential prognostic tool

RA is characterised, as well as by clinical variability, by a high degree of biological heterogeneity at the tissue level. Whether distinct synovial features translate into specific clinical phenotypes and predict diverse clinical outcomes is still largely unknown.

So far, a limited number of studies have evaluated the potential impact of synovial lymphocytic aggregates on predicting prognosis in inflammatory arthritis, and the majority of them have been performed in patients with established RA. Relatively few studies were performed in patients captured at an early phase. Also, the interpretation of the existing data is complicated by a number of issues. Firstly no specific criteria are consistently used between studies to define synovial aggregates histologically and as such the definition of ELS has been variously used to refer to aggregates of differing size and/or expressing different specific immunological cells (e.g. FDC, T cells, B cells). Further comparing data from cross sectional studies including patients with varying a) disease duration, b) radiographic damage and c) treatment exposure has inherent methodological discrepancies. Finally, excessive prevalence of synovial tissue from the knee, as is the case with a number of studies, may introduce systematic bias, by including those patients with the most aggressive disease. [288] Therefore, this concept requires further examination in a large scale prospective study of early arthritis patients naïve to therapy.

A key question to address is the utility of pathobiology in informing therapeutic intervention/response. This is particularly relevant for bDMARD which, despite having dramatically changed the natural history of RA, still shows an unexplained

range of inter-individual variability in the response. In particular, TNFα antagonists are the most widely used bDMARD and have been in routine use for the longest. TNFα is an important mediator of the induction and maintenance of secondary lymphoid organs [377,378], so the capacity for modulation of synovial aggregates by TNFα inhibitors has the potential to dissect the pathophysiology of the disease as well as identification of biomarkers. Recent studies have produced somewhat conflicting results. A first study from Canete *et al* investigated the relationship between response to anti-rheumatic treatment including TNFα inhibitors and the identification of large B cell aggregates on pre-treatment arthroscopic synovial biopsy in 86 patients: data suggested that synovial lymphoid neogenesis was an independent negative predictor of response to therapy, and response to anti-TNFα treatment was associated with the regression of lymphocytic aggregates. [330] However, a subsequent report from Klaasen in which synovial biopsies were performed on 97 patients prior to commencing treatment with Infliximab, found that the presence of lymphocytic aggregates was rather a positive predictor of response at 16 weeks. [324] There are a number of reasons for such discrepancies between the two groups including patient cohorts differing in terms of past drug exposure, disease duration and a non homogeneous definition of synovial aggregates used within each study.

Another study identified pre-treatment levels of synovial TNFα as a positive predictor of response to treatment. [379] A subsequent study of 143 patients supported these results showing that TNFα expression within the synovial sub-lining explained about 10% of the variance in response to therapy; further when a clinical prediction model incorporating disease activity and TNFα expression within

the synovial sub-lining was formulated, 17% of variance in response to therapy could be explained. [380]

Finally, previous works suggested an association between the number of sub-lining synovial macrophages and joint damage in RA. The importance of macrophages in disease pathogenesis is indirectly suggested by the beneficial effect of strategies aimed at targeting macrophage-derived cytokines like TNFα, IL-1, and IL-6. As previously mentioned, it has been observed that sub-lining macrophages (CD68sl) are a sensitive biomarker for response to treatment in patients with RA, specifically there was a significant correlation between the change in the number of macrophages and the change in DAS28. [64] The results suggest that synovial sublining macrophages might be used for the evaluation of new anti-rheumatic treatments. [381]

Thus although at present we remain some distance from personalised healthcare, these studies demonstrate proof of concept that in the long term the integration of synovial pathobiology into clinical prediction models may prove to be a useful clinical tool. It seems likely that any robust prediction model in the future will have to incorporate multiple pathobiological in addition to clinical, serological and imaging tools.

1.10 SUMMARY AND HYPOTHESIS

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease of unknown origin associated with considerable morbidity and early mortality. Its clinical course is highly variable, ranging from self-limiting to severe disease, which can lead to joint damage, chronic disability and loss of function.

Advances in recent years, such as early intervention, treat to target strategy and introduction of biologic drugs have revolutionised the natural history of the disease and improved outcome and quality of life of patients. [1] However treating patients with RA remains challenging. To start an appropriate treatment at an individual level, it is crucial to stratify patients into accurate diagnostic and prognostic categories. Current prediction models have limited value in identifying progressive disease in early arthritis. [382] This suggests there is a high need for identification of new biomarkers of disease prognosis and response to therapy.

Importantly, as well as clinical variability, RA shows high biological heterogeneity. In the past decades there has been increasing recognition that synovial tissue is the primary site of inflammation. Nonetheless limited data has evaluated synovial cellular biomarkers of disease prognosis including response to therapy, with most studies of small cohorts in long standing disease.

The aim of this thesis is to examine the hypothesis that distinct pathotypes can be identified in the rheumatoid synovium at both an early and longstanding stage, which associate with specific clinical phenotypes and moreover can predict response to therapy.

The specific aims are to identify whether:

1. in an early RA cohort, the synovial pathotype significantly associates with a specific clinical phenotype;
2. in an early RA cohort, the synovial pathotype predicts clinical response to csDMARD treatment;
3. in an established RA cohort, the synovial pathotype predicts clinical response to the TNFα inhibitor Certolizumab pegol.

Chapter 2 : MATERIALS AND METHODS

2.1 GENERAL PROTOCOLS

This thesis will focus on two separate cohorts of patients with RA: an early RA cohort (experimental chapters 3 and 4) and an established RA cohort (experimental chapter 5).

The first cohort is represented by 63 consecutive patients with early RA (disease duration <12 months) who were csDMARD and corticosteroid treatment naïve recruited at Barts Health National Health Service (NHS) Trust as part of a multicentric, Medical Research Council (MRC)-funded study named Pathobiology of Early Arthritis Cohort (PEAC), http://www.peac-mrc.mds.qmul.ac.uk. The aim of PEAC study was to generate an extensive cohort of patients with early inflammatory arthritis with linked clinical, serological, radiological and pathobiological data. This data includes gene expression profiling, proteomics, metabonomics, serum and synovial fluid cytokine and chemokine analysis, radiographic and ultrasound imaging, synovial tissue analysis, and detailed clinical phenotyping. This to facilitate understanding the spectrum of molecular and cellular pathways underlying disease heterogeneity and identifying potential biomarkers of disease progression and treatment response.

Inclusion criteria and exclusion criteria of PEAC are showed in Table 2.1.

INCLUSION CRITERIA	EXCLUSION CRITERIA
- ≥ 18 and years of age	- Biopsy contraindicated (e.g. patients taking anticoagulants)
- Evidence of active arthritis DAS28>2.6	- Serious underlying medical disorders (e.g. active infection, cancer, end stage renal or hepatic disease)
- At least one swollen joint	
- Able to give written informed consent	

Table 2.1: PEAC study: inclusion/exclusion criteria
Abbreviations: DAS28= 28 joint count-Disease Activity Score

The study received local ethical approval (REC 05/Q0703/198) and all patients gave written informed consent.

The second cohort includes 28 consecutive patients with established RA (mean disease duration 6.2 years) fulfilling 2010 ACR/EULAR criteria, who had failed a course of at least two csDMARD, of which one represented by MTX, who were eligible for anti-TNFα therapy according to NICE guidelines [http://www.nice.org.uk/nicemedia/pdf/CG79NICEGuideline.pdf]. These patients were part of a monocentric observational cohort of a study conducted at Barts Health NHS Trust sponsored by Queen Mary University of London (QMUL) named "Clinical responsiveness to anti-TNFα therapy and modulation of synovial lymphoid structures and B cell function in RA- An exploratory open label prospective study in RA -(CLIP-Cert)". The study received local ethic approval and written informed consent was obtained by all patients.

2.2 CLINICAL ASSESSMENT

Clinical assessment included the following parameters: smoking status; disease duration; number of swollen joints (SJ) and tender joints (TJ); a 100- cm scale Visual Analogue Score for Global Health (VAS-GH) assessed by patient; measure of disease activity assessed by 28 joint count-Disease Activity Score (DAS28), a universally validated composite measure incorporating TJ, SJ, ESR and VAS-GH [383]. DAS28 scores range from 0 to 10 scale, with DAS28 < 2.6 representing remission, ≤ 3.2 low disease activity, < 3.2 and ≤ 5.1 moderate disease activity, and > 5.1 high disease activity status. [384]

Response to treatment was evaluated according to EULAR response criteria, a fully validated tool based on DAS28 changes (Table 2.2). [385] Three categories of response were inferred: good response, moderate response and no response. For the purpose of this thesis, clinical response was defined as achievement of either good or moderate EULAR response. The impact of the disease on physical performance was evaluated by using the Health Assessment Questionnaire (HAQ), which is the most widely used method to assess functional status. [386]

DAS28 at endpoint	Improvement in DAS or DAS28 from baseline		
	> 1.2	> 0.6 and ≤ 1.2	≤ 0.6
≤ 3.2	Good	Moderate	None
> 3.2 and ≤ 5.1			
> 5.1			

Table 2.2: The EULAR response criteria based on DAS28
Abbreviations: DAS28= 28 joint count-Disease Activity Score.

2. 3 LABORATORY ASSESSMENT

Laboratory assessment included routine biochemistry (full blood count, liver function test, urea and electrolytes), ESR, CRP. Presence of IgM-RF determined by nephelometry (positive > 20 UI/ml) and ACPA antibodies determined by second-generation enzyme-linked immunosorbment assay (positive > 9 UI/ml) was assessed in all patients at baseline. These tests were performed by the NHS laboratories at The Royal London Hospital, Barts and The London Trust.

2.4 RADIOGRAPHIC ASSESSMENT

Digital antero-posterior radiographs (XR) of the hands and feet were obtained at baseline.

For the early RA cohort only, XR were repeated at 12 months in order to evaluate radiographic progression. Anonymised images were scored according to the van der Heijde modified Sharp score (ShSS). [387] The total ShSS score is the result of the sum of two parameters: joint erosions (JE) and joint space narrowing (JSN) for hands and feet. The ShSS method includes, in each hand, 16 areas for JE and 15 areas for JSN, and, in each foot, 6 areas for JE and 6 areas for JSN. The erosion score per joint of the hands can range from 0 to 5. The erosion score per joint of the foot range from 0 to 10. The Joint space narrowing score is identical for hands and feet ranging from 0 to 4. Erosions and joint space narrowing are always scored and no judgment should be made on whether they are due to rheumatoid process or to osteoarthritic lesions. The total replacement or the chirurgical fusion of a joint automatically assigns a maximal erosion and narrowing score to that joint. Maximal total erosion score of the hands is thus 160. Maximal

total erosion score of the feet is 120. Maximal total erosion score (hands and feet) is 280. Maximal total narrowing score in the hands is 120. Maximal total narrowing score in the feet is 48. Maximal total narrowing score (hands and feet) is 168. Maximal total Sharp/van der Heijde score is 448.

Images have been acquired at Barts and The London Hospitals radiology department, and scored by Dr Frances Humby, a trained reader who was blinded for clinical and histopathological data. 10% of images were scored by a second independent observer, Dr Annette van der Helm-van Mil, in order to determine intra-class correlation coefficients (ICC) for reliability.

Mean total ShSS, mean JSN and mean JE between synovial pathotype groups were examined. In addition the mean changes between baseline and 12 months in the three radiographic parameters were evaluated. Radiological progression was defined as any increase (≥ 1 point) in the ShSS or in any of its individual component (JSN and SE).

For the established RA cohort, the ShSS scoring was not applicable, and the erosive status was assessed according to the evaluation of an experienced musculoskeletal radiologist at Barts and The London Hospitals.

2.5 ULTRASONOGRAPHIC ASSESSMENT

US images were acquired by myself using a GE Logic 9 ultrasound machine with a two-dimensional M12L transducer (14MHz). Standard longitudinal images of the 1^{st}-5^{th} MCP (Figure 2.1) and midline, radial and ulnar views of both wrists joints (Figure 2.2) were acquired as previously described. [287] The ultrasound examination was performed at room temperature (20-22 °C) and the operator

used minimal probe pressure after applying ultrasound gel on the surface of the skin and maintaining a distance of at least 1 mm of gel between the probe and the contact surface. The colour box was adjusted to cover the region of interest (ROI). The colour priority, dynamic range and persistence were set high. The power Doppler settings were adjusted to the lowest permissible pulse repetition frequency to maximize sensitivity, and maximum colour gain was set just below noise level.

In summary, the setting was as follows: Grey Scale- Frequency 14 MHz; Power Doppler - Frequency 7.5 MHz, Gain 41, PRF 1.4 kHz, Wall Filter 127 Hz.

Images were stored in DICOM format. The digitally stored images were then transferred to a processing program (ImagePro) and subsequently underwent semi-quantitative (SQ) assessment for synovial thickening and power doppler signal by two trained independent assessors- Dr Nora Ng and Dr Ilias Lazarou- who were blinded to clinical and histopathological data. The scoring was performed in compliance with the Outcome Measures in Rheumatology (OMERACT) US synovitis scores. [388] An illustration and detailed description of the scoring system is shown on Figure 2.3. Briefly, a 0-3 SQ scoring system was applied to quantify STUS and PDUS at the single joint level (10 MCP joints and midline view of both wrists) for each patient, with ST-G0 and PD-G0 representing absence and ST-G3 and PD-G3 representing maximum grade of ST/PD respectively. The total STUS and the total PDUS scores were then calculated by summing up the respective scores for each of the 12 joints examined, therefore ranging from a minimum value of 0 to a maximum value of 36. In this thesis, by STUS and PDUS scores I will refer to the total STUS and PDUS scores.

10% of images were scored by a second independent observer, Dr Stephen Kelly, showing good to excellent ICC.

The variation of STUS and PDUS scores between baseline and 6 months were calculated. US remission was defined as total PDUS score ≤ 1.

Figure 2.1: Ultrasound scan of longitudinal view of the 2nd left metacarpophalangeal joint

Figure 2.2: Ultrasound scan of longitudinal view of the left wrist mid-line

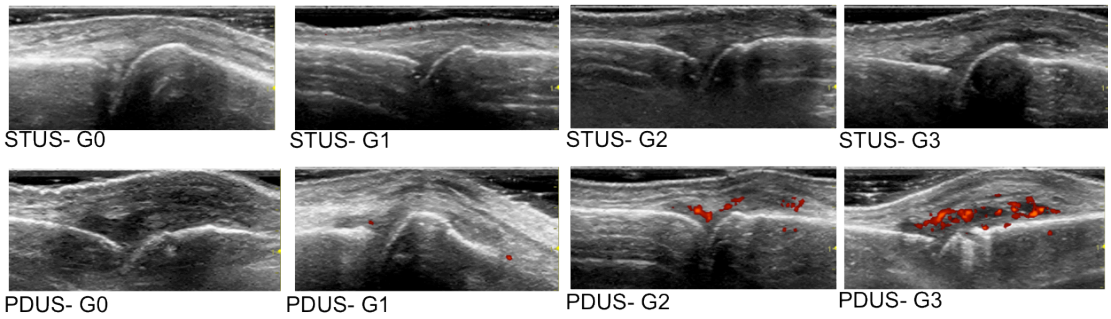


Figure 2.3: Semi-quantitative assessment for synovial thickening and power doppler activity according to the OMERACT definition
Abbreviations: STUS= synovial thickening ultrasound; PDUS= power doppler ultrasound.
Score range from grade 0 (G0) to grade 3 (G3) for each parameter.

Scoring for STUS
STUS-G0= No hypoechoic synovial thickening.
STUS-G1= Minimal hypoechoic synovial thickening (filling the angle between the periarticular bones, without bulging over the line linking tops of the bones).
STUS-G2= Hypoechoic synovial thickening bulging over the line linking tops of the periarticular bones but without extension along the bone diaphysis.
STUS-G3= Hypoechoic synovial thickening bulging over the line linking tops of the periarticular bones and with extension to at least one of the bone diaphyses.

Scoring for PDUS
PDUS-G0= No flow in the synovium.
PDUS-G1= Up to 3 single spots or up to 2 confluent spots or 1 confluent spot + up to 2 single spots.
PDUS-G2= Vessel signals in less than half of the area of the synovium (< 50%).
PDUS-G3= Vessel signals in more than half of the area of the synovium (> 50%).

Courtesy of Dr Stephen Kelly

2.6 ULTRASOUND-GUIDED SYNOVIAL BIOPSY

All patients underwent an US-guided synovial biopsy of a clinically inflamed joint before starting treatment. In order to facilitate the joint selection for biopsy, US assessment pre-biopsy was utilized, and specifically the level of STUS. The STUS scoring was done real time by mutual agreement between the ultrasonographer and a second assessor (Dr Nora Ng or Dr Ilias Lazarou). The subsequent decision tree was followed: medium or large joint with STUS= 3; small/medium/large joint with STUS ≥ 2; medium/large joint with STUS ≥ 1; small joint of any ST grade. [287]

Biopsies were performed by trained Rheumatologists within the Rheumatology Department at The Royal London Hospital: Dr Stephen Kelly, Dr Frances Humby, Dr Nora Ng, Dr Arti Mahto, Dr Ilias Lazarou.

1-3 mls of local anaesthetic was injected into the soft tissues up to the joint capsule, visualized under US guidance. A further 2-5 mls of local anaesthetic (1% lignocaine) was instilled into small joints, 10-15 mls into large joints.

A 16/14G Quick-Core® Biopsy Needle (Cook medical, Limerick, Ireland) was then placed within the joint capsule and a longitudinal US image used to detect the needle and guide it to an appropriate pre-determined site for biopsy. A minimum of 6 biopsies were taken and immediately fixed in 4% paraformaldehyde for subsequent paraffin embedding. [287]

Examples of US guided synovial biopsy of the second MCP and wrist are shown on Figure 2.4 and Figure 2.5 respectively.

Figure 2.4: Ultrasound-guided synovial biopsy of the second MCP joint
Abbreviations: MCP= metacarpophalangeal.
Courtesy of Dr Stephen Kelly

Figure 2.5 : Ultrasound-guided synovial biopsy of the wrist
Courtesy of Dr Stephen Kelly

2.7 HISTOPATHOLOGICAL ANALYSIS

Histopathological analysis was performed by Dr Rebecca H. Hands, Mrs Vidalba Rocher and Dr Alessandra Nerviani in the central laboratory at Experimental Medicine and Rheumatology, William Harvey Institute, Queen Mary University of London.

Following US-guided biopsy, synovial tissue was immediately fixed in 4% paraformaldehyde for later paraffin embedding. A minimum of 2 samples were embedded per block. After paraffin embedding, three 5-µm-thick sections from each biopsy specimen obtained 50 µm apart underwent routine haematoxylin and eosin (H&E) staining and if intact lining layer identified graded as suitable for further histopathological assessment. If no intact lining layer was visible, a further three 5-µm-thick sections at least 50 µm apart were cut and examined. If no visible lining layer was seen, tissue was counted as ungraded. [289]

In order to define the predominant histological pattern within each sample the presence and/or size of lymphocytic aggregates within each section was determined and graded according to a modified, previously published grading system [319] with grade 1 (G1) aggregates displaying a radial cell number between 2 and 5 cells, grade 2 (G2) between 6 and 10 cells and grade 3 (G3) greater than 10 cells, with G2 and G3 defining large sized lymphocytic aggregates.

Additionally in order to determine the degree of immune cell infiltration sequentially formalin-fixed paraffin-embedded tissue sections were deparaffinised and rehydrated through graded ethanol solutions and then stained for B cells (CD20), T cells (CD3), macrophages (CD68) and plasma cells (CD138) as previously described. [389] Once rehydrated antigens were retrieved with Target Retrieval

Solution (DAKO). The following primary antibodies were used: mouse anti-human CD20 (L26, DAKO), rabbit anti-human CD3 polyclonal (A0452, DAKO), mouse anti-human CD68 (PGM1, DAKO), mouse anti-human CD138 (MI15, DAKO). Appropriated biotinylated secondary antibodies were used.

Sections then underwent SQ (0-4) for CD3, CD20, CD68 lining (CD68l), CD68 sub-lining (CD68sl) and CD138, with a score of 0 representing minimal cellular expression and a score of 4 high cellular expression. Two trained readers – Dr Rebecca E. Hands and Mrs Vidalba Rocher- who were blinded with regard to clinical details scored all sections. When scores between the two readers did not match, the scoring was resolved by mutual agreement.

Samples were classified as: 'Lymphoid' based on the presence of large lymphocytic aggregates (G2/G3); 'Myeloid' based on the absence of G2/G3 aggregates but abundant CD68sl (2-4 SQ); 'Pauci-immune' (PI) characterised by absence of G2/G3 aggregates as well as scarce/absent CD68sl (0-1 SQ).

2.8 STATISTICAL ANALYSIS

Statistics will be described in each experimental chapter according to the type of evaluations and analysis performed. In this paragraph I will briefly enounce the general principles followed.

Continuous variables were expressed as mean (standard deviation, SD), ordinal variables were expressed as median (interquartile range, IQR).

Demographics and clinical characteristics between groups were compared using Chi-Square test for qualitative variables or Kruskal Wallis test for quantitative variables, as appropriate.

Spearman correlation coefficients were used for semi-quantitative variables and Pearson correlation coefficient for continuous variables.

Logistic regression analysis was performed to estimate if the synovial pathotype was an independent predictor of response to therapy after 6 months in the PEAC and after 3 months in the CLIP-Cert cohort. The relevant adjusting variables were sequentially selected in the modelling process.

Missing values in the dataset were imputed using multivariate imputation by chained equations (MICE). [390] The multiple imputations were implemented using R 3.0.2 package 'mice'. Statistical analyses were performed using SPSS (IBM version 21.0 for Mac). A p-value < 0.05 was considered statistically significant.

Chapter 3 : THE CHARACTERISTICS OF THE SYNOVIUM IN AN EARLY RHEUMATOID ARTHRITIS COHORT

3.1 INTRODUCTION

The presence of distinct molecular and cellular phenotypes within the synovium has attracted increased attention since its recognition as the primary site of inflammation in RA. However whether specific synovial pathotypes are associated with distinct disease subtypes (e.g. seronegative versus seropositive, erosive versus non erosive, responding versus resistant pattern) remains unknown. Furthermore there is a growing body of evidence to suggest that early RA may represent a distinct phase of the pathobiological disease process, but the notion of 'early disease' remains purely clinical and its temporal cut-off arbitrary.

Synovial inflammation has been identified at a microscopic level prior to the onset of clinically apparent synovitis in RA patients.[93] However whether the cellular and/or molecular characteristics of the synovium are distinct at distinct disease phases is currently unknown. Such a hypothesis has been examined in a number of different cohorts with somewhat conflicting results. Some authors postulated that, whenever differences in the features of the synovium are observed, they do not appear to depend on disease duration but rather on the level of disease activity at that specific time-point, reflecting the status of local inflammation rather than a specific chronological phase of the disease process. [391] Examining synovial samples obtained within the first 4 weeks of symptoms onset, Schumacher and Kitridou found no major differences compared to tissue samples from patients with longer disease duration. [392] Similarly, Smeets *et al* reported that the degree of cellular infiltration and T-cell activation were similar in early versus long-standing RA. [393] A subsequent study comparing 31 samples from patients with early RA to 35 samples from patients with established disease failed

to identify major differences with regard to lining layer hyperplasia, infiltration by monocytes/macrophages, lymphocytes, polymorphonuclear cells and mast cells, and expression of IL-6 and TNFα. [394] Conversely, Singh JA *et al,* analysing synovial specimens from 8 patients with very early RA (< 6 weeks duration), detected profound differences compared with an established disease infiltrate: specifically they could not see any presence of lymphoid aggregates in early RA, and observed a cellular infiltrate limited to the superficial area of the synovium only, consisting mainly of perivascular T cells with a limited number of B cells, along with a scarce expression of neo-vascularity. [395]

More recently, gene expression profiling technologies have represented an important step-up in synovial tissue analysis, allowing further dissection of the molecular heterogeneity of RA synovium at different time-points. Genomics studies were able to detect marked differences in synovial RNA expression between patients with early and established RA. For example, a study comparing 4 early versus 4 long-standing RA identified specific gene clusters and molecular signatures expressed differently in the early and long-standing synovium, suggesting the prevalence of distinct pathophysiological mechanisms during different phases of the disease. [396]

To date, given such amount of controversial and often opposite results, it is difficult to draw any reliable conclusion about distinct stages of synovial inflammation and intrinsic pathobiological features. However, the majority of these studies are affected by several methodological discrepancies where multiple biases had been operating. Firstly, most studies are small in size and based on cross-sectional rather than prospective data. Secondly, they often include analyses

of arthroplastically obtained synovial tissue and large joint arthroscopic biopsies, which may have introduced bias by selecting more severe patients. [288] This is of particular concern when evaluating early arthritis cohorts, where small rather than large joints are most commonly involved.[287] It is also relevant to note that synovial pathobiology can also be modulated by therapeutic intervention. Not less importantly, there is a high variability in terms of synovial tissue extraction techniques (e.g., tissue obtained during joint replacement surgery, arthroscopy, needle biopsies, US-guided minimally invasive biopsy), as well as synovial tissue analysis methods, histological classifications and scoring systems to quantify the degree of cellular infiltrate. Collectively, these factors represent important caveats of synovial tissue studies so far. Therefore further examination of large-scale, systematic prospective studies of synovial membrane are needed to better understand the pathophysiological mechanisms involved in RA, particularly in early RA patients who are naïve to any anti-rheumatic treatment.

Moreover US has been long recognised as an accurate reflection of disease activity in inflammatory arthritis, and actually PDUS has proved to be more sensitive than clinical assessment in detecting joint inflammation. [220,397] However little is known about the relationship between US and synovial pathology. A few studies have shown that US measures of inflammation reflect features of histological synovitis, but these were mostly performed examining large joints of patients at end-stage disease. [398-401] Little work has been done to describe this relationship in early RA populations.

Koski *et al* examined PDUS in relation to histopathological features of 44 synovial sites including large as well as small joints, bursae and tendon sheaths. [402]

Biopsy samples were obtained by using US-guided synovial biopsy. The authors found no significant correlations between the extent of synovial inflammation and the amount of power Doppler signal assessed on a SQ scale. An association was found with the presence/absence of PD signal only. Conversely, in a recent study by Andersen *et al* evaluating 81 needle arthroscopy samples from hands and wrists of 29 RA patients, a significant association between the doppler colour fraction and the extent of inflammation, including correlation with specific cell subsets CD68 and CD3, was found. [403]

A recent publication examined the relationship between US synovitis and synovial vascularity including the expression of angiogenic factors, lymphangiogenic factors and cellular mediators of inflammation in a cohort of early RA patients prior to therapeutic intervention. An US-guided synovial biopsy of the supra-patella pouch was performed in 12 patients. PDUS showed a good correlation with angiogenic factors such as vascular endothelial growth factor- A (VEGF-A), Angiopoietin 2 and Tie-2. A significant correlation was also found for both STUS and PDUS with the pro-inflammatory cellular and cytokine profile specifically TNFα, IL-6, IL-1β, podoplanin (a lymphatic endothelial marker) and CD68, providing consistent validity in its use as an objective assessment of synovial inflammation. [404]

3.2 AIMS AND OBJECTIVES

The aim of this chapter was to determine whether synovial pathotype significantly associates with clinical phenotype in an early RA treatment naïve cohort. The specific objectives were to:

1) describe the pathological characteristics of the synovial membrane in a treatment naïve early RA cohort;
2) evaluate whether the pathological features of the synovium were associated with distinct phenotypes with regard to clinical features, antibody status, radiological and ultrasound findings.

3.3 MATERIALS AND METHODS

3.3.1 Study population

63 consecutive patients with early RA (disease duration <12 months, fulfilling 2010 ACR classification criteria) who were csDMARD and corticosteroid treatment naïve were recruited at Barts Health NHS Trust as part of the multi-centred MRC-funded Pathobiology of Early Arthritis Cohort (PEAC), http://www.peac-mrc.mds.qmul.ac.uk. The study has been described in details in the Material and Methods section (Chapter 3).

3.3.2 Patient assessment

Patients underwent baseline clinical and laboratory assessment, XR of hands and feet, US of 10 MCPs and wrists and US-guided synovial biopsy prior to starting csDMARD treatment, as described in details in Chapter 3.

3.3.3 Tissue sample collection and histopathological analysis

Detailed methods of biopsy procedure, tissue collection, histopathological analysis and pathobiological scoring have been described in Chapter 3.

Synovial samples were then stratified in one of the three pathobiological groups: 'Lymphoid', based on the presence of large lymphocytic aggregates (G2/G3); 'Myeloid', based on the absence of G2/G3 aggregates and predominant expression of and CD68sl 2-4 SQ; 'Pauci-immune' (PI) characterised by the absence of G2/G3 aggregates and scarce/absent CD68sl (0-1 SQ).

3.3.4 Statistical analysis

Continuous variables were expressed as mean (+/- SD), ordinal variables as median (+/- IQR).

Demographics and characteristics of patients across the three histopathological groups were compared using Chi-Square test for qualitative variables or Kruskal Wallis test for quantitative variables, as appropriate.

Spearman correlation coefficient was used for semi-quantitative variables and Pearson correlation coefficient for continuous variables.

A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS (IBM version 21.0 for Mac).

3.4 RESULTS

3.4.1 Characteristics of patients

The demographic and clinical characteristics of patients at baseline are shown on Table 3.1.

Among the 63 patients, 73% were female. Mean age was 50.4±17.5. 24.6 % were current smokers. The mean duration of symptoms onset was 5.4±3.1 months. Mean ESR was 39.8±26.5 and mean CRP 18.4±28.2. 67.7% were

seropositive for RF and 68.2% for ACPA. Mean TJC was 12.0±7.2 and mean STJ was 8.0±5.3. The mean DAS28 was 5.8±1.2, falling in a high disease activity range. Mean HAQ was 1.63±0.67.

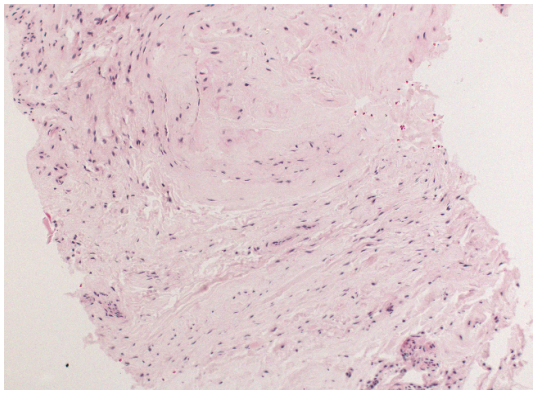
Of the 63 US-guided synovial biopsy procedures, 50 (79.4%) were performed on small joints (40 wrists, 8 MCPs, 2 PIPs) and 13 (20.6%) on large joints (12 knees, 1 elbow).

	N=63 (100%)
Female, n (%)	46 (73%)
Age (years), mean ± SD	50.4 ± 17.5
Onset (months), mean ± SD	5.4 ± 3.1
Smoking, n (%)	15 (24.6%)
ESR (mm/h), mean ± SD	39.8 ± 26.5
CRP (mg/L), mean ± SD	18.4 ± 28.2
RF +, n (%)	42 (67.7%)
ACPA +, n (%)	43 (68.2%)
TJ (28 joints), mean ± SD	12.0 ± 7.2
SJ (28 joints), mean ± SD	8.0 ± 5.3
VAS-GH (0-100 mm), mean ± SD	66.0 ± 25.9
DAS28, mean ± SD	5.8 ± 1.2
HAQ, mean ± SD	1.63 ± 0.67
ShSS, mean ± SD	14.1 ± 16.4
JSN, mean ± SD	12.2 ± 12.9
JE, mean ± SD	1.9 ± 4.2

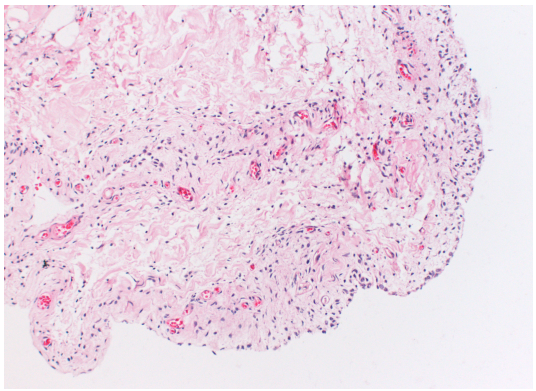
Table 3.1: Demographics and clinical characteristics of PEAC patients at baseline
Abbreviations: ACPA= anti-cyclic citrullinated proteins antibodies; CRP= C-reactive protein; DAS28= 28 joint count-Disease Activity Score; ESR= erythrocyte sedimentation rate; HAQ= Health Assessment Questionnaire; JE= joint erosions; JSN= joint space narrowing; RF= rheumatoid factor; ShSS= van der Heijde modified Sharp score; SJ= swollen joints; TJ= tender joints; VAS-GH= Visual Analogue Score for Global Health.

3.4.2 A Lymphoid synovial pathotype significantly associates with higher levels of ESR and seropositivity for RF and ACPA antibodies

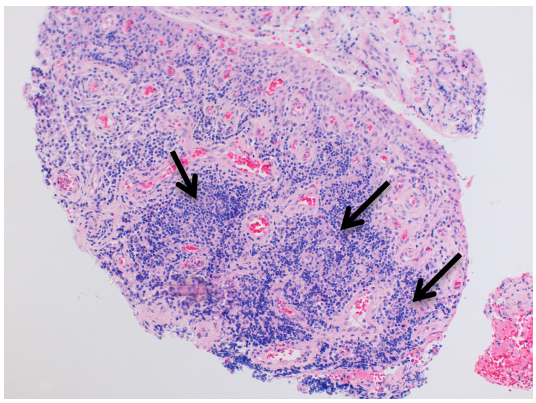
A Lymphoid pattern was found in 24 patients (38.1%), a Myeloid pattern in 19 (30.2%) and a PI pattern in 20 (31.7%), Figure 3.1.



A. Pauci-immune pattern (31.7% of patients)



B. Myeloid pattern (30.2% of patients)



C. Lymphoid pattern (38.1% of patients)

Figure 3.1: Representative images of the three histopathotype patterns
Pauci-immune (A), characterised by absence of lymphocytic aggregates and scarce/absent sublining macrophages (SQ 0-1); Myeloid (B) characterised by absence of lymphocytic aggregate and presence of sublining macrophages (SQ 2-4); and Lymphoid (C) characterised by presence of large lymphocytic aggregates grade 2-3 (black arrows) resembling ectopic lymphoid tissue.
Pictures from the PEAC biobank. Courtesy of Prof. C. Pitzalis.

In order to determine if there were significant differences in disease characteristics between patients classified as Lymphoid, Myeloid or PI, patients were segregated into each pathological group and mean differences in demographics compared (Table 3.2).

	Pauci-immune N=20 (31.7%)	Myeloid N=19 (30.2%)	Lymphoid N=24 (38.1%)	p value
Female, n (%)	16 (80.0%)	11 (57.9%)	19 (79.2%)	0.20
Age (years), mean ± SD	45.3 ± 15.7	55.3 ± 19.6	50.8 ± 16.7	0.17
Onset (months), mean ± SD	6.3 ± 3.4	4.5 ± 2.0	5.3 ± 3.5	0.23
Smoking, n (%)	5 (27.8%)	6 (31.6%)	4 (16.7%)	0.49
ESR (mm/h), mean ± SD	30.8 ± 25.9	35.9 ± 24.9	50.3 ± 25.6	0.01
CRP (mg/L), mean ± SD	20.6 ± 43.4	14.4 ± 16.1	20.0 ± 19.9	0.08
RF +, n (%)	11 (57.9%)	10 (52.6%)	21 (87.5%)	0.02
ACPA +, n (%)	12 (60%)	10 (52.6%)	21 (87.5%)	0.03
TJ (28 joints), mean ± SD	11.8 ± 7.7	11.4 ± 6.9	12.7 ± 7.3	0.74
SJ (28 joints), mean ± SD	6.9 ± 5.8	8.1 ± 5.4	8.7 ± 5.0	0.25
VAS-GH (0-100 mm), mean ± SD	59.1 ± 30.9	66.1 ± 27.5	71.6 ± 18.9	0.51
DAS28, mean ± SD	5.5 ± 1.4	5.7 ± 1.2	6.3 ± 0.8	0.10
HAQ, mean ± SD	1.65 ± 0.75	1.68 ± 0.54	1.57 ± 0.73	0.98
ShSS, mean ± SD	11.6 ± 15.9	17.2 ± 21.6	13.4 ± 11.0	0.45
JSN, mean ± SD	10.5 ± 13.5	14.0 ± 15.9	12.1 ± 9.5	0.46
JE, mean ± SD	1.1 ± 2.9	3.2 ± 6.5	1.3 ± 1.9	0.17

Table 3.2: Characteristics of patients compared across the histopathotype groups
Kruskal Wallis test or Chi-Square test, as appropriate.
Abbreviations: ACPA= anti-cyclic citrullinated proteins antibodies; CRP= C-reactive protein; DAS28= 28 joint count-Disease Activity Score; ESR= erythrocyte sedimentation rate; HAQ= Health Assessment Questionnaire; JE= joint erosions; JSN= joint space narrowing; RF= rheumatoid factor; ShSS= van der Heijde modified Sharp score; SJ= swollen joints; TJ= tender joints; VAS-GH= Visual Analogue Score for Global Health.

Although there were no significant differences in demographics including gender, age, smoking status and clinical characteristics including disease duration, TJ, SJ, VAS-GH, DAS28, HAQ among groups, mean ESR was significantly higher in the Lymphoid group (50.3 ± 25.6, compared with 35.9 ± 24.9 in the Myeloid and 30.8 ± 25.9 in the PI, p=0.01). Additionally when patients were further segregated into those who were RF+/- and ACPA +/- there was a significantly higher number of RF+ (PI=57.9%, Myeloid=52.6%, Lymphoid=87.5%, p=0.02) and ACPA + (PI=60%, Myeloid=52.6% Lymphoid=87.5%, p=0.03) in patients categorised in the Lymphoid group. Further a significant difference was observed on a direct pairwise comparison of groups between Lymphoid and PI (p= 0.03 for RF and p=0.03 for ACPA) and between Lymphoid and Myeloid (p=0.01 and p=0.01) however this was not seen between Myeloid and PI (p= 0.74 and 0.64). This data again supports the significant relationship between a Lymphoid pathotype and circulating disease specific autoantibodies. Testing for multiple comparisons was not performed as the purpose of this work was looking at how specific variables affected the three groups rather than proving that the three groups were generally different.

Given the significant association between tobacco smoking and ACPA positivity we also went on to evaluate whether smoking habit was differently distributed within the three histopathotype groups, but this was not the case (PI=27.8%, Myeloid=31.6% and Lymphoid=16.7%, p=0.49).

In order to determine whether the presence of large synovial aggregates was significantly associated with synovial infiltration of T cells, B cells and/or macrophages, synovial samples were stratified into each synovial pathotype group and mean SQ scores of each cell population evaluated. The presence of synovial

aggregates was significantly associated with a higher infiltration of CD3, CD20, CD68L, CD68SL and CD138 cells ($p<0.01$, Table 3.3).

	Pauci-immune N=20 (31.7%)	Myeloid N=19 (30.2%)	Lymphoid N=24 (38.1%)	p value
CD3	0 (0-0)	1 (1-2)	3 (2-4)	<0.01
CD20	0 (0-0)	1 (0-1)	3 (3-4)	<0.01
CD68l	1 (0-1)	2 (1-2)	3 (2-4)	<0.01
CD68sl	1 (0-1)	2 (2-3)	3 (3-4)	<0.01
CD138	0 (0-0)	0 (0-1)	3 (3-4)	<0.01

Table 3.3: Synovial pathotype is associated to the extent of tissue cellularity
Values are expressed as median (interquartile range) of the semi-quantitative (0-4) score. Kruskal Wallis test.
Abbreviations: CD3, T cells; CD20, B cells; CD68l, lining macrophages; CD68sl, sub-lining macrophages; CD138, plasma cells.

3.4.3 Synovial pathotype does not discriminate between clinically assessed levels of disease activity

No association was found between the synovial pathotype and clinical features including TJ, SJ, VAS-GH, level of disease activity expressed by DAS28. The mean DAS28 was 5.8 ± 1.2, reflecting high disease activity status that was consistent through all three histopathological groups in the cohort.

An association between the synovial pathotype and the functional status measured by HAQ has not been detected either.

3.4.4 An aggregate synovial pathotype associates with significantly higher levels of ultrasonographic disease activity

Next in order to determine if US-synovitis scores correlated with laboratory and clinical markers of disease activity, baseline STUS and PDUS scores were correlated with clinical and laboratory parameters. Good quality US images were obtained and scored for 60 patients. PDUS score significantly correlated with mean ESR ($r=0.31$, $p=0.01$), CRP ($r= 0.45$, $p<0.01$) and DAS28 ($r=0.30$, $p=0.01$), whilst a significant correlation with STUS score was only demonstrated for CRP ($r=0.33$, $p=0.01$). This data indicates that in early RA synovial PDUS may be a better indicator of disease severity than STUS, which as previously suggested may associate more strongly with disease duration. [405,406]

Furthermore as clinical assessment has been demonstrated to be less reliable than US assessment for the detection of synovitis [226,366], in order to determine if synovial pathotype associated with more severe disease assessed ultrasonographically, baseline STUS and PDUS scores of patients segregated into

Lymphoid, Myeloid and PI pathotypes were compared. There was no significant difference in STUS scores between either pathotype but a significantly higher PDUS score was demonstrated in patients with a Lymphoid (11.1 ± 9.5) compared to Myeloid (7.3 ± 8.8) and PI (4.7 ± 7.9) pathotype (p=0.04), Table 3.4 supporting the concept that an aggregate pathotype associates with more active disease at the US level.

Finally in order to determine whether mean US scores significantly correlated with degree of immune cell infiltration, SQ CD20, CD68l, CD68sl and CD138 scores were correlated with STUS and PDUS scores. I found no significant correlations with STUS score, meanwhile a significant correlation with PDUS score was observed (Table 3.5) in line with previously reported data. [403] These observations support the role of PDUS as a sensitive indicator of synovial pathology.

	All N=63 (100%)	Pauci-immune N=20 (31.7%)	Myeloid N=19 (30.2%)	Lymphoid N=24 (38.1%)	p value
STUS	19.7 ± 13.1	19.2 ± 11.0	18.8 ± 13.8	20.8 ± 14.7	0.89
PDUS	8.0 ± 9.1	4.7 ± 7.9	7.3 ± 8.8	11.1 ± 9.5	0.04

Table 3.4: Ultrasound features at baseline compared across histopathotype groups
Values are expressed as mean ± SD. Kruskal Wallis test.
Abbreviations: PDUS= power doppler ultrasound; STUS= synovial thickening ultrasound.

	CD3	CD20	CD68l	CD68sl	CD138
STUS	-0.03	0.05	-0.14	0.07	0.09
PDUS	0.26*	0.32*	0.20	0.32*	0.38**

Table 3.5: Power doppler ultrasound scores correlate significantly with the degree of synovial immune cell infiltration
Values are expressed as Spearman Rho coefficient, *= significant at <0.05. **= significant at <0.01.
Abbreviations: CD3, T cells; CD20, B cells; CD68l, lining macrophages; CD68sl, sub-lining macrophages; CD138, plasma cells; PDUS= power doppler ultrasound; STUS= synovial thickening ultrasound.

3.4.5 The synovial pathotype does not associate with a specific erosive pattern at baseline

Finally, in order to determine whether synovial pathotype associated with specific radiographic damage we stratified patients into each synovial pathotype and evaluated whether there were significant differences in the total ShSS score along with either JSN or erosions. Good quality XR images suitable for subsequent scoring were obtained for 54 patients. No significant differences were demonstrated in either the total ShSS or its individual components.

3.5 DISCUSSION

The existence of distinct histological patterns has been previously observed in the RA synovium, however longitudinal data from patients with early RA naïve to treatment are scarce. Within the early RA cohort described in this work, three major pathotypes have been identified: Lymphoid (38%), characterised by presence of large lymphocytic aggregates resembling ectopic lymphoid tissue; Myeloid (30%), characterised by absence of lymphocytic aggregates however presence of abundant inflammatory cells in a non-aggregate distribution, including sublining macrophages; and Pauci-immune (32%) characterised by overall scarce/absent immune cell infiltrate. Previous studies have suggested that the presence of a distinct pathotype may confer specific phenotypic characteristics. However, the role of the synovial pathotype in determining the clinical phenotype including response to treatment is still controversial.

First of all, it is worth noting that the proportion of a Lymphoid pathotype in this early RA cohort is comparable to that observed in arthroscopic synovial

biopsies from patients with established RA. [323] This suggests that the acquisition of lymphocytic aggregates resembling ectopic lymphoid tissue represents an early step of RA pathogenesis rather than a feature acquired at later stages as was previously suggested [407], supporting a potential role in an early local peripheral breach of tolerance. In agreement with these findings, Cantaert *at al* reported the presence of lymphoid aggregates in 31% of RA patients, with no difference between early and established disease. [408]

The results presented herein demonstrate a number of novel findings namely that in early RA a Lymphoid synovial pathotype i) significantly associates with ESR and seropositivity for both RF and ACPA; ii) significantly associates with mean PDUS score which represents a reliable measure of synovitis. Collectively this data suggests that a Lymphoid pathotype associates with a more aggressive clinical phenotype. Furthermore the significant association between such pathotype and seropositivity for RF and ACPA strongly supports the concept that synovial aggregates are immunologically competent as demonstrated by previous ex vivo data. [325] Since most studies failed to observe an association between antibody status and presence of large aggregates in long-standing RA [323,408], our data suggests that this could be a distinct characteristic of early RA. In addition the significantly higher levels of CD138+ plasma cells in tissue with lymphocytic aggregates strongly suggests in situ B cell differentiation.

The results presented within this cohort are important and robust for a number of key reasons. Firstly patients were treatment naïve early RA patients from currently one of the largest reported pathobiological early RA series. This is critical as it is well recognised that concomitant treatment with csDMARD and/or biologic

agents modulate synovial pathobiology [324,330], a factor that was not controlled for in previous studies. Secondly the application of the minimally invasive technique of US-guided synovial biopsy to this cohort ensured that patients were recruited irrespective of the need for significant large joint involvement resulting in an unbiased cohort. In former studies, biopsies have been mainly obtained from arthroplasty knee joints with by definition end-stage disease and may not be well representative of typical histopatology changes characteristic of the early phase of the disease.

In previous works, an association between the histological features of the synovial membrane and the clinical measures of disease activity have been reported. [409,410] I could not detect any significant association between synovial pathotype and clinical features including TJ, SJ, VAS-GH, DAS28 and HAQ. This suggests that, at least in the early phase of the disease, clinical features of patients showing different pathotypes may be indistinguishable. Nonetheless, the failure to detect differences could be due to low statistical power related to the small sample size, which may have led to a classic type 2 statistical error.

Importantly herein I provide evidence that a Pauci-immune synovial pathotype is seen in over 30% of patients. This pathotype, although characterised by a low level/virtually absence of an immune cells infiltrate and despite being associated to a lower level of autoantibodies and acute phase reactants, is associated with clinically active disease. Recent evidence emerging from transcriptomic analysis suggests that different pathotypes segregate with different cellular expression, specifically the Lymphoid and Myeloid pattern are mostly represented by lymphocytes and macrophages respectively, meanwhile the Pauci-immune type

segregates with a predominant fibroblast expression. [411] This is notable particularly in view of recent advances in understanding the unique role of synovial mesenchymal stromal cells, and especially synovial fibroblasts, as powerful modulators of the immune response. [76,81] Indeed stromal cells, traditionally considered as structural, homeostatic supportive and immunological inert cells, are proved to be actively involved in innate and adaptive immune response. Ultimately they can be viewed as tissue-resident modifiers, functioning through crosstalk with cells of hematopoietic origin. [412]

Townsend et al propose a schematic model summarizing different cellular contribution to RA pathogenesis, highlighting the fact that, while biological features such as the expression of inflammatory markers and autoantibodies may fluctuate over different pathotype groups, the clinical features may be similar (Figure 3.2). [411]

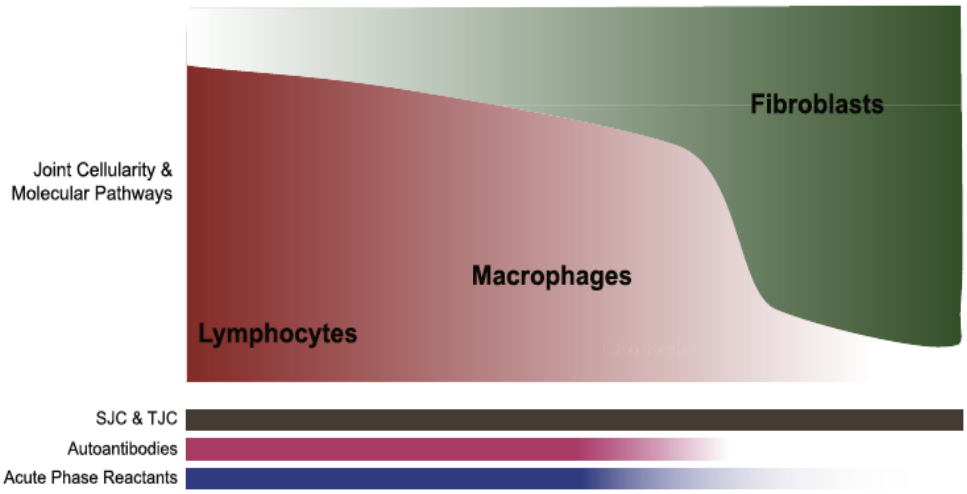


Figure 3.2 : Differential cellular contributions to disease pathogenesis in the rheumatoid synovium

This schematic representations shows that different predominant cell subsets (lymphocytes, macrophages and fibroblasts) may characterise the heterogeneity of inflamed synovium in rheumatoid arthritis, although these may represent continuous rather than discrete, mutually exclusive subsets. These distinct synovial pathotypes may translate into different expression of autoantibodies and acute phase reactants (higher in the lymphocyte-predominant pattern and minimal/virtually absent in the fibroblast-predominant pattern) despite similar clinical features expressed as number of swollen and tender joints.

Abbreviations: SJC= swollen joint count, TJC= tender joint count.

From [411], with permission.

In this work a significant role for lymphocytic aggregates in disease pathogenesis is also supported by the significant association with PDUS measures of synovitis. Given that this is an early arthritis cohort, the lack of association with STUS is not surprising, as PDUS has been demonstrated to be a more sensitive and responsive measure of disease activity with the STUS/PDUS ratio generally reduced in early RA compared to chronic disease. [405,406] These results reinforce the general notion that the extent of STUS synovitis correlates with disease duration, probably reflecting the level of persistent inflammation and subsequent fibrotic changes; in contrast, the presence of PDUS signal seems to be independent of disease duration and appears a specific indicator of active inflammation at any given time-point. Crucially as the US assessment was performed immediately prior to synovial biopsy the confounding effect of daily variability in the synovial micro-flow was minimized. [413]

An important limitation of US-based studies is the lack of agreement on the standard joint set that should be adopted for assessing US synovitis in RA patients. The 12 joint set applied to this cohort has been utilized in previous case series, therefore is not without validation. [414] Notably, I have observed a significant correlation between measures of US synovitis expressed as PDUS activity and measures of global inflammation and disease activity including ESR, CRP and DAS28, which strength the validity of the joint set selection. However in addition to considering whether local synovial pathobiology reflects global disease activity a crucial future research question is whether this relationship persists at the single joint level.

Despite demonstrating a significant association between a Lymphoid pathotype and high levels of ESR, ACPA/RF positivity and PDUS synovitis suggesting a more severe clinical phenotype this was not associated with worse radiological joint damage at baseline. There are a number of reasons for this, firstly: by definition, due to inclusion of patients with a short-disease duration (mean disease duration 5.4 months), only a few patients are expected to present with structural change on plain XR. Indeed, disease duration may be too short for the XR to be sensitive enough to detect erosive changes and other radiological features such as periarticular osteoporosis have been suggested to be more sensitive indicators of disease severity. [415] It could be speculated that more sensitive techniques such as US and MRI may have been able to detect early structural changes, and possibly differences among histopathological groups. Future studies integrating an imaging modality such as MRI, with the capacity to correlate synovial pathobiology with early markers of joint damage such as cartilage loss, bone marrow oedema and erosions are likely to yield significant results.

Finally, in this study I have not specifically looked for fibroblasts expression on the IHC analysis, and therefore it cannot be inferred that the PI pathotype equates to a predominant fibroblastic group. However a detailed genome-wide transcriptomic analysis in currently ongoing to explore such hypothesis.

Chapter 4 : SYNOVIAL PATHOTYPE PREDICTS CLINICAL AND ULTRASOUND OUTCOME IN EARLY RHEUMATOID ARTHRITIS

4.1 INTRODUCTION

Predicting the clinical course of RA is challenging as wide inter-individual variation in clinical outcome and response to treatment exists. It has been estimated that major differences in the outcome range 10-12 fold over 10 years. [416] Treating patients early, intensively and to target is instrumental in maximizing the clinical benefits of treatment as well as achieving effective prevention of functional decline. [237] The introduction of new drugs and specifically biologic agents has also represented a major therapeutic advance over the last two decades, however high costs of these drugs limit their use to patients who have already failed first-line treatment with csDMARD therapy. This approach may lead to treatment delay for patients with more aggressive disease. Indeed it has been suggested that earlier intervention with biologic therapy in combination with MTX could be indicated in a proportion of patients. [417,418] On the other hand, an intensive approach is probably not necessary for all patients with a new diagnosis of RA, and giving all patients maximal treatment would not only incur high healthcare costs but also expose individuals to unnecessary risk of side effects. [419]

Unfortunately, it is difficult to predict which patients will respond adequately to various treatment regimens, with current treatment algorithms based on sequential therapies derived from trial and error rather than risk-stratification approach.

A number of clinical, laboratory, and genetic markers have been associated with severe prognosis, particularly the presence of RF and ACPA. [420] However, none of these markers alone or in combination are sufficiently sensitive to identify

patients who will progress into worst outcome and able to inform treatment decisions on individual basis. [97,421]

In the recent years, the recognition of the synovium as the primary site of inflammation has led to its evaluation as a biomarker of prognosis and therapeutic response in RA with the underlying premise that biological heterogeneity observed at the tissue level may affect subsequent clinical outcome and response to treatment. Such an approach offers the potential to dissect RA pathogenesis and therefore may facilitate the discovery of new disease pathways and therapeutic targets, and ultimately the development of new generation target-specific drugs.

Previous studies have suggested that the degree of sublining CD68+ macrophage infiltration at baseline is associated with progressive joint damage. [422] Furthermore, sublining layer macrophages have been confirmed as a robust biomarker of clinical response to both csDMARD and biologic agents. [64,332] Further data have also suggested that clinical response to anti-TNF therapy may be related to synovial tissue inflammation levels prior to treatment, providing proof of concept that synovial biomarkers may be used to predict the response to treatment. [324,330]

However, little attention has been given to the potential prognostic role of the synovium in early RA, where a limited number of confounding factors impacts on the features of synovial infiltrate with the potential therefore to robustly evaluate synovial pathobiological biomarkers.

According to the 2016 update of the EULAR recommendations for the management of early RA [423], csDMARD should be started as soon as the diagnosis

of RA is made. A trend towards a clinical improvement should be observed within the first 3 months of therapy, and the achievement of the treatment target should be attained within 6 months. However it is recognised that achievement of clinical response, and even stable clinical remission, is not sufficient to ensure favourable outcome over time. The first evidence of a significant disconnection between clinical composite indices of remission and imaging findings was demonstrated by Brown *et al*. [220] By performing US and MRI of 2nd-5th MCPs and wrist of the dominant hand in 107 patients who were in stable clinical remission following csDMARD treatment, they found that both US and MRI showed persistent subclinical synovitis in a considerable proportion of patients: in particular, PDUS signal was present in 43% and MRI signal in 96% of asymptomatic patients with clinically normal joints. Of most importance, in a subsequent study of 102 patients, the persistence of imaging-detected synovitis correlated with long-term structural damage with an OR of 12. [226] Similar findings have been confirmed by other authors. [221,424] Further studies showed that the presence of PDUS activity was an accurate predictor of flare/short term relapse. [369,406,425]

It has been estimated that up to 50% of patients with RA will develop structural damage over the first year [147] and more than 80% within the second year [148], and that this is associated with decline in functional capacity and quality of life. However, not all patients progress in the same way. Clinical heterogeneity of RA is in fact further confirmed by discovery of coexisting "slowly" and "rapidly" progressive phenotypes. [426] Several potential predictors of structural damage have been identified, including both biological (genetic markers, autoantibodies, acute phase reactants, cartilage markers, bone markers, synovial tissue markers) and

clinical indicators (pre-existing joint damage, number of swollen and tender joints, measures of disease activity, body mass index). However none have been reliably sensitive or specific to be used in routine clinical practice. The role of synovial pathobiology in predicting joint damage progression in RA has also been examined with generally discrepant results. Klimiuk and co-workers observed more advance joint destruction in patients with lymphoid aggregates compared to those without, supporting a direct contribution of these structures in sustaining tissue damage. [329] van de Sande, in a prospective analysis of 93 patients with early arthritis, concluded that the presence of a lymphoid pattern at baseline was not predictive of the development of persistent and erosive arthritis after 2 years of follow-up. [314] However to date there has been no examination of the role of synovial pathobiology in predicting radiographic damage in therapy naïve early RA patients.

It was on this background that I aimed at exploring, in a treatment naïve early RA cohort, whether the synovial pathotype at baseline would be able to inform clinical outcome after 6 months of csDMARD therapy. The key question is whether synovial heterogeneity translates into diverse clinical outcomes, and specifically whether a synovial biopsy at onset could predict response to csDMARD therapy at 6 months. Furthermore, given this dissociation between clinically-detected and imaging-detected synovitis reported across studies, and provided that US seems to reflect more faithfully the intrinsic pathobiological process of the disease compared to clinical tools, an important additional aspect of my work will be to evaluate STUS and PDUS scores change at 6 months, and especially the differences in the magnitude of the change across the three histopathological groups. Finally,

the relationship between the baseline pathotype and the progression of radiographic damage at 12 months will be evaluated.

4.2 AIMS AND OBJECTIVES

The aim of this work is to test the hypothesis that distinct histological pathotypes within the synovial membrane in a treatment naïve early RA cohort provide characteristic prognostic implications, and specifically to determine whether the presence of a specific synovial pathotype at baseline:

1) can predict clinical response to treatment at 6 months as determined by DAS28 changes and achievement of EULAR response criteria;
2) it is associated with significant modulation of STUS and PDUS scores at 6 months;
3) it is associated with radiographic joint damage progression at 12 months.

4.3 MATERIALS AND METHODS

4.3.1 Study population

63 consecutive early RA patients (disease duration <12 months, fulfilling 2010 ACR/EULAR classification criteria [4], csDMARD and corticosteroids naïve) recruited as part of the PEAC study at Barts Health NHS Trust were included within the study. The baseline characteristics of the study population have been described in Chapter 3 (Tab 3.1).

4.3.2 Clinical assessment

Patients were assessed at baseline and then 3 monthly for 12 months. At each visit, clinical and laboratory assessment was performed as described in Materials and Methods. The DAS28 was utilized to assess disease activity and guide therapeutic management based on a treat to target approach (set target DAS28 < 2.6, defining clinical remission).

4.3.3 US guided synovial biopsy

At baseline, all patients underwent an US-guided synovial biopsy of one actively inflamed joint as previously described (Chapter 2, paragraph 2.6). At 6 months, a second biopsy of the same joint was also obtained.

4.3.4 Histopathological analysis

According to the features of the synovium, patients were stratified into three synovial pathotype groups: PI, Myeloid or Lymphoid and the degree of immune cell infiltration with CD20, CD3, CD68 and CD138 determined semi-quantitatively (0-4).

4.3.5 Ultrasonographic and radiographic assessment

Ultrasonographic assessment was performed at baseline and at 6 months follow-up. Radiographic assessment was performed at baseline and at 12 months follow-up. Please refer to Chapter 2 (paragraphs 2.4 and 2.5) for detailed description of the methodology applied.

4.3.6 Treatment

Immediately following US-guided synovial biopsy, patients were commenced on combination with MTX (target dose 20 mg/weekly) plus SSZ (target dose 2 g/daily) or HCQ (target dose 400 mg/daily). Patients on MTX also received ≥ 5 mg Folic acid/weekly according to standard guidelines. Oral PRED was started at physician discretion at the dose of ≤ 7.5 mg/daily. Concomitant treatment with NSAIDs and intra-articular injections of corticosteroids were allowed for all treatment groups at physician discretion. Given that this was an observational rather than interventional study alternative therapeutic regimens were also permitted e.g. Leflunomide or csDMARD monotherapy.

Clinical assessments were subsequently performed at 3 month intervals with a treat-to-target approach to treatment escalation with an aim for clinical remission (DAS28 < 2.6). In case of side effects, treatment was continued at the lowest tolerated dosage.

Patients failing csDMARD therapy at 6 months were considered for biologic agent in accordance with NICE guidelines http://www.nice.org.uk, and managed in line with best practice.

4.3.7 Outcome measures

Primary outcomes assessed after 6 months of therapy were:

1) mean change in disease activity based on DAS28;
2) proportion of patients achieving EULAR response at 6 months.

Secondary outcomes were:

1) mean change in mean STUD and PDUS scores at 6 months;

2) mean change in mean ShSS, JSN and JE scores at 12 months.

4.3.8 Statistical analysis

Continuous variables were expressed as mean (+/- SD), ordinal variables as median (+/- IQR).

Comparisons of characteristics between PI, Myeloid and Lymphoid groups were made using Chi-Square test for qualitative variables or Kruskal Wallis test for quantitative variables, as appropriate.

Spearman correlation coefficients were used for semi-quantitative variables and Pearson correlation coefficients for continuous variables.

Logistic regression analysis was performed to estimate if the synovial pathotype was an independent predictor of response to therapy after 6 months. The relevant adjusting variables were sequentially selected in the modelling process. The results were expressed as OR with 95% confidence intervals (95% CI).

The statistical analysis for clinical outcomes (primary outcome) was performed on all patients; the statistical analysis for US and XR scores change (secondary outcomes) were performed on patients with a complete dataset only.

Missing values in the dataset were imputed using multivariate imputation by chained equations (MICE). The multiple imputations were implemented using R 3.0.2 package 'mice'. [390] All other statistical analyses were performed using SPSS (IBM version 21.0 for Mac). All tests were 2-sided and a p-value < 0.05 was considered statistically significant.

4.4 RESULTS

4.4.1 The presence of a baseline Lymphoid pathotype predicts clinical response to csDMARD therapy

55 patients (87.3%) received csDMARD combination (42 MTX+ SSZ, 13 MTX + HCQ) and 7 (11.1%) csDMARD monotherapy (2 MTX, 1 SSZ, 4 HCQ). In one case (1.6%) csDMARD were not started as clinically contraindicated. 41 subjects (65%) received oral PRED at the dose of 7.5 mg or less daily. Triple therapy (MTX + SSZ + HCQ) was started at 3 months in 5 patients who were still presenting with high disease activity (DAS28 > 5.1) despite initial combination with MTX, SSZ and PRED. 4 patients were started on biologics (3 anti-TNFα, 1 Rituximab) as presenting with high persistent disease activity at 6 months.

In order to determine whether clinical response to treatment was influenced by synovial pathotype, I first evaluated the difference in DAS28 between 6 month and baseline and compared across the three histopathological groups.

The mean DAS28 at baseline was 5.8 ± 1.2 and at 6 months was 3.8 ± 2.0, with a lower DAS28 in the Lymphoid compared to the Myeloid and PI, although this did not reach statistical significance (PI=4.3 ± 2.2, Myeloid=3.6 ± 1.9 and Lymphoid =3.4 ± 1.8, p=0.38). The mean change (Δ) in DAS28 between the two time-points was -2.0 ± 2.0, with a larger fall in DAS28 in patients characterised as Lymphoid (PI=-1.1 ± 1.9, Myeloid=-2.1 ± 2.1, Lymphoid=-2.8 ± 1.6, p=0.01) Figure 4.1.

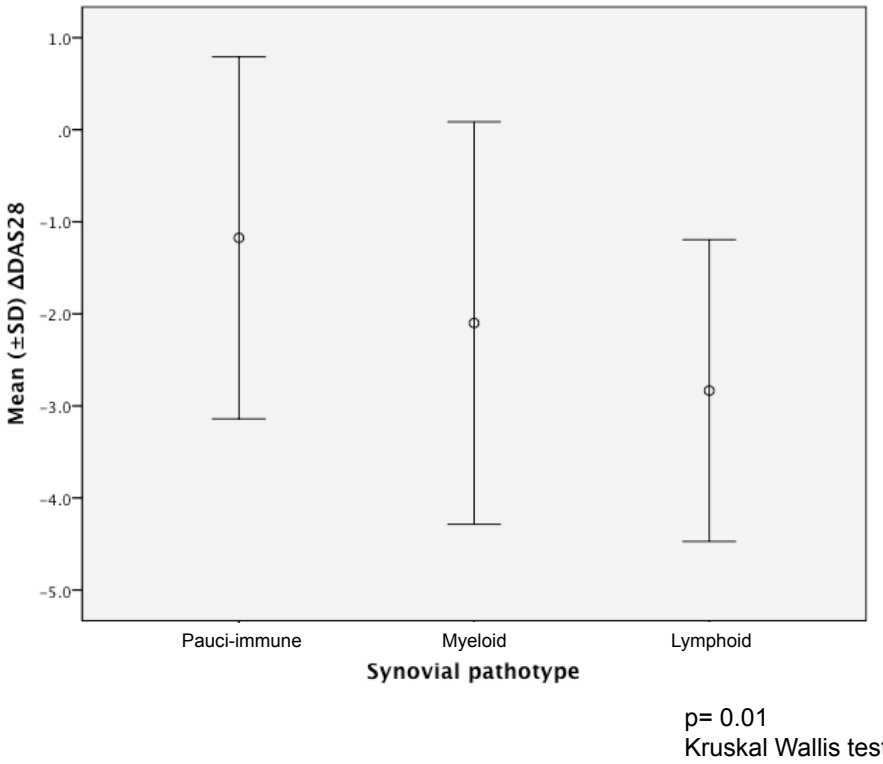


Figure 4.1: The Lymphoid histopathotype is associated with a higher fall in DAS28 at 6 months

Abbreviations: ΔDAS28= mean change in 28 joint count-Disease Activity Score; SD= standard deviation.

Importantly, when Student's test was applied it resulted that the change in DAS28 was not significantly affected by its baseline values ($t= 1.61$, $p=0.11$).

There was a significant correlation between the ΔDAS28 and baseline expression of CD3 ($r=-0.32$, $p=0.01$), CD20 ($r=-0.36$, $p<0.01$) and CD68sl ($r=-0.34$, $p=0.01$), indicating that the higher the cellular infiltrate at baseline, the higher the magnitude of the change in disease activity.

In addition the rate of patients that were EULAR responders was calculated, and the proportion of responders in each pathotype group determined. A total of 43/63 patients (68.3%) were good/moderate responders, with a significantly higher number of patients responding within the Lymphoid group (PI: Myeloid: Lymphoid = 45%: 73.7%: 83.3%, $p=0.02$) Figure 4.2.

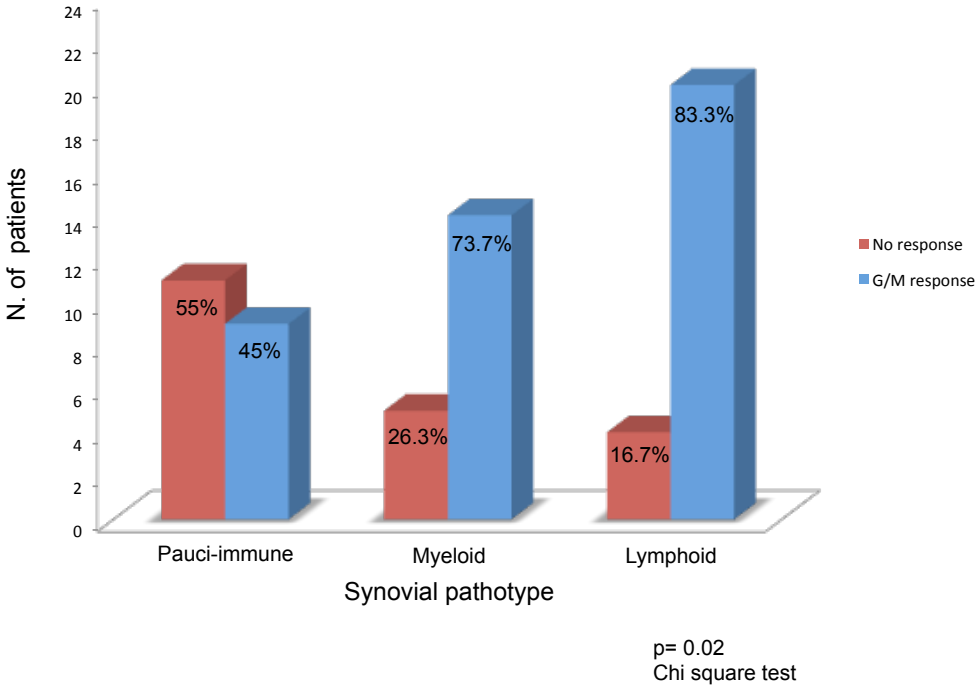


Figure 4.2: A significantly higher number of patients with a Lymphoid pathotype achieved a EULAR response at 6 months vs Myeloid or Pauci-immune
Abbreviations: G/M= good/moderate response according to EULAR response criteria.

Furthermore when determining whether there were significant differences in numbers of patients achieving EULAR good response within each pathotype I found that it was significantly lowest in the PI group (PI: Myeloid: Lymphoid = 35.0%: 55.6%: 54.2%, p=0.06). However the numbers of patients reaching low disease activity (DAS28 ≤ 3.2) and remission (DAS28 < 2.6) was not significantly different across the three groups (p=0.19 and p=0.93, respectively).

I also found that baseline expression of CD3, CD20 and CD68sl within the synovium was significantly higher in patients who achieved EULAR response compared to non responders: CD3= 2.0 (1.0-3.0) *vs* 1.0 (0.0-2.1), p=0.04; CD20= 1.7 (0.0-3.0) *vs* 0.2 (0.0-1.7), p= 0.049; CD68sl= 3.0 (2.0-4.0) *vs* 1.0 (1.0-3.0), p=0.01.

I then performed a logistic regression analysis to assess the factors/covariates predicting EULAR response. Variables included in the analysis were: gender, age, ESR, CRP, DAS28, ACPA and RF status, use of oral steroids, PDUS, ShSS, synovial pathotype. Each variable was tested for the association with EULAR response using univariate logistic regression analysis, then multivariate logistic regression analysis was performed using a backward stepwise method to select independent predictors. Results are showed in Table 4.1. Age and synovial pathotype were identified as significant predictors of EULAR response, however synovial pathotype was the only predictor selected by all five imputed datasets for missing data.

	Univariate model			Multivariate model		
	OR	CI 95%	p value	OR	CI 95%	p value
Gender	1.733	0.485-6.199	0.39			
Age	0.981	0.951-1.012	0.22	0.900	0.835-0.970	<0.01
ESR	1.004	0.984-1.025	0.68			
CRP	0.999	0.980-1.019	0.94			
DAS28	0.901	0.576-1.409	0.64			
ACPA+	0.455	0.145-1.422	0.17			
RF+	0.600	0.196-1.834	0.37			
Oral steroids	0.724	0.241-2.173	0.56			
PDUS	1.050	0.978-1.128	0.17			
ShSS	1.011	0.973-1.050	0.57			
Synovial pathotype	2.531	1.250-5.125	0.01	7.714	2.046-29.085	<0.01

Table 4.1: Association between baseline characteristics and EULAR response at 6 months using univariate logistic regression analysis and multivariate logistic regression analysis

Abbreviations: ACPA= anti-cyclic citrullinated proteins antibodies; CI= confidence interval; CRP= C-reactive protein; DAS28= 28 joint count-Disease Activity Score; ESR= erythrocyte sedimentation rate; OR= odds ratio; PDUS= power doppler ultrasound; RF= rheumatoid factor; ShSS= van der Heijde modified Sharp score.

4.4.2 Fall in sublining macrophage number significantly correlates with fall in DAS28 at 6 months.

A second biopsy of the same joints was obtained for 49 patients at 6 months. Pre- and post-treatment paired biopsy data were available for 44 patients.

I went onto to examine whether changes in SQ sublining macrophage number between baseline and 6 months correlated significantly with changes in DAS28. Paired synovial samples and clinical outcome data were available for 44 patients. In line with previously reported data a significant correlation was observed (Spearman's r= 0.36, p=0.01) (Figure 4.3).

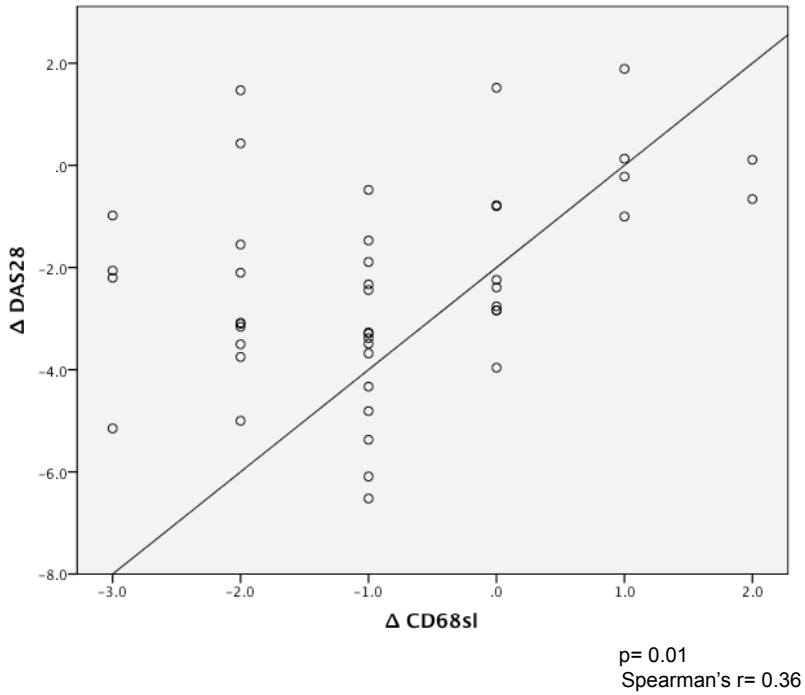


Figure 4.3: Correlation between change in clinical disease activity and change in semi-quantitative sublining macrophages between baseline and 6 months
Abbreviations: ΔCD68sl= mean change in semi-quantitative sublining macrophages; ΔDAS28=mean change in 28 joint count-Disease Activity Score.

4.4.3 A numerically larger decrease in power doppler ultrasound synovitis scores was seen in patients with a baseline Lymphoid pathotype

Next US response was examined. STUS and PDUS scores at 6 months were correlated with laboratory and clinical markers of disease activity.

Paralleling results from baseline scores, PDUS at 6 months correlated with ESR ($r=0.40$, $p<0.01$), CRP ($r=0.72$, $p<0.01$) and DAS28 ($r=0.29$, $p=0.04$), meanwhile STUS correlated with CRP only ($r=0.39$, $p<0.01$).

In order to determine whether STUS and PDUS scores changed in line with clinical response, the variation of STUS and PDUS scores between baseline and 6 months were correlated with the variation of DAS28 in the cohort of 47 patients in which paired US data was available: there was no significant association with mean change in DAS28 and mean change in STUS score ($r=0.16$, $p=0.28$) meanwhile a significant correlation with mean change in PDUS score was demonstrated ($r=0.46$, $p<0.01$) Figure 4.4.

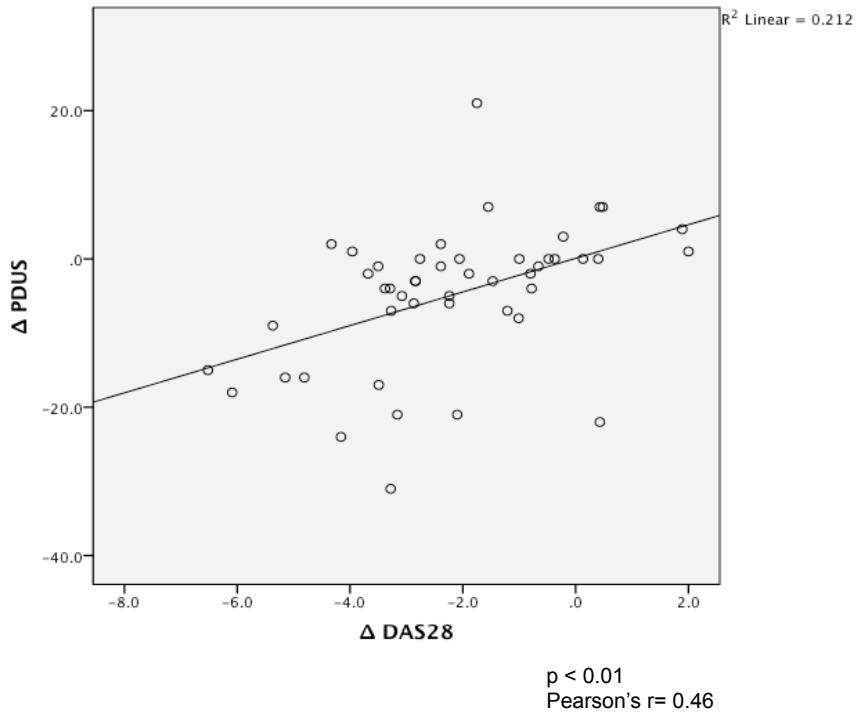


Figure 4.4: Fall in PDUS parallels fall in DAS28 after 6 months
Abbreviations: ΔDAS28= mean change in 28 joint count-Disease Activity Score; ΔPDUS= mean change in power doppler ultrasound score.

Next given the importance of the modulation of US synovitis in determining outcome for patients with RA [427], the mean change in STUS and PDUS scores between baseline and 6 month follow-up was compared across the three synovial pathotype groups. There was a trend toward larger mean fall in PDUS score in the Lymphoid group compared to the other two groups (PI: Myeloid: Lymphoid = -1.1±8.5: -4.6±8.0: -9.3±10.6, p=0.08) (Figure 4.5).

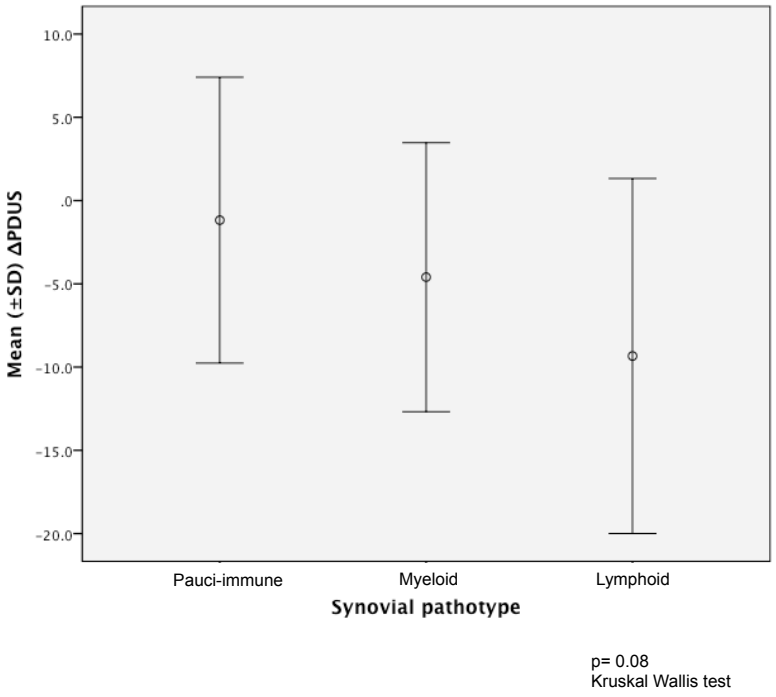


Figure 4.5: The presence of a Lymphoid histopathotype is associated with a trend in higher fall in PDUS

Abbreviations: ΔPDUS= mean change in power doppler ultrasound score.

I also observed a significantly larger mean fall in the Lymphoid vs PI group in PDUS score (p=0.02) but not in STUS score (p=0.91). Given the reverse ratios between STUS and PDUS in early versus late disease [405,406] and previous data supporting a critical role for PDUS but not for STUS in predicting disease progression [226] such a result is not surprising and provides further support for the concept that a Lymphoid pathotype is associated with more robust response to csDMARD in early RA.

Finally I also went on to stratify patients into patients with a PDUS score ≤1 and >1 and examined whether there were differences in numbers between the three pathotypes. Overall 53.2% of patients showed PDUS score ≤ 1 and no significant difference across the three pathotype groups was observed (p=0.49).

4.4.4 The synovial pathotype at baseline is not associated with significant radiographic progression at 12 months

Finally in the group of 42 patients with a paired set of radiographic films at both baseline and 12 months, the relationship between synovial pathotype at baseline and joint damage progression was examined. Importantly the characteristics of patients without paired data did not differ from the whole group.

Mean total ShSS, mean JSN and mean JE between synovial pathotype groups were examined. In addition the mean change (Δ) between baseline and 12 months in the three radiographic parameters was evaluated and compared across the three histopathotypes. No association between the histopathotype and the erosive status at 12 months (mean ShSS, JSN and JE scores) as well as the radiological progression (any change in either ΔShSS, ΔJSN or ΔJE scores between

12 months and baseline) was observed. The proportion of patients who showed a progression in the ShSS was numerically higher in the Lymphoid group (35%) vs the Myeloid group (20%) *vs* the PI group (10%), although these results did not reach statistical significance (p=0.30). This is likely due to a number of factors including the small number of patients with paired radiographic data.

A significant correlation between serum levels of RF at baseline with JE score at 12 months (r=0.46, p<0.01) and ΔJE (r=0.43, p<0.01) was found, confirming wide reports of RF as a strong predictor of radiological progression. [354,420,428-430] These results may reinforce the importance of the relationship between the antibody titre at baseline and the joint damage during the course of RA, as reported by other authors [431,432] and also highlighted by 2010 ACR/EULAR criteria. [4] Intriguingly, a significant correlation between the number of synovial CD138 and the delta JE score was observed (r=0.35, p=0.02), suggesting that the plasma cells in the synovium could be a direct source of pathogenetic antibodies. Although significant correlations between structural progression and the ACPA levels at baseline have been widely reported in the literature [353,356,433,434], I was not able to confirm similar results herein.

Notably in the patients that achieved US remission (PDUS score ≤1) at 6 months versus those that did not, the JE score was significantly lower at 12 months (1.2 ± 2.8 *vs* 3.5 ± 6.2, p=0.01) in addition to a significantly lower increase in JE between baseline and 12 months (0.0 ± 0.2 *vs* 0.3 ± 0.4, p=0.04). This result is in line with previous data demonstrating that persistent US synovitis associates with worse radiological outcomes. [221]

4.5 DISCUSSION

RA is characterised by biological and clinical variability, including heterogeneous response to treatment. The extent and the features of the immune cell infiltrate within the synovium are variable among individuals with RA since the early stage of the disease. In some patients, a scarce/absent infiltration is observed; in others the cellular infiltrate is randomly diffused within the joint tissue; in a few more, B and T cells are organised into lymphocyte aggregates that may exhibit germinal centre–like features. At present, it is unknown whether there is a differential response to specific treatments between patients exhibiting these distinct pathogenic subsets.

Herein I have investigated the relationship between the pre-treatment synovial pathotype and the clinical response to first line csDMARD in a cohort of 63 early RA patients after 6 months of therapy.

The results revealed a highly significant relationship between the presence of a specific synovial pathotype at baseline and the primary clinical response to csDMARD. When the synovial pathotype was added into a prediction model along with demographics, clinical and serological parameters, and after adjusting for potentially confounding variables by multivariate analysis, the presence of a Lymphoid pathotype resulted as a strong independent predictor of response to therapy. Conversely, the predominance of a Pauci-immune histological pattern in the non-responder group suggests that this subset may be associated with treatment resistant early RA. Because the patients with a Lymphoid pathotype were presenting with features of more severe disease such as higher level of ESR and higher rate of seropositivity for ACPA and RF at baseline, it could be

postulated that these patients were likely to have received more intensive treatment. That was not the case in the present cohort, as the treatment was not different across categories. This because all patients had similar degree of disease activity expressed by DAS28 score and were therefore managed intensively. Moreover, the use of steroids was similar across the 3 histopathotype categories and has not been selected as a potential predictor of response in the logistic regression model.

Previous data has suggested that a Lymphoid pathotype can predict response to Infliximab in csDMARD resistant RA patients. [324] However this is the first report in a treatment naïve early RA cohort examining response to csDMARD. The majority of patients were treated with MTX (90%), with 68.3% (43/63) patients achieving a moderate/good EULAR response at 6 months. Given that MTX remains the gold standard first-line treatment for patients with RA [253] despite a poorly understood mechanism of action [435], elucidating the heterogeneous responses to treatment and moreover identification of a robust prognostic biomarker of response would prove invaluable. Clinical response to MTX therapy is multifactorial, and several key elements such as pharmacogenetics, pharmacokinetics and pharmacodynamics are involved, which have not been explored in the present work. In particular, it has been reported that several genetic variants have been associated to MTX response, such as polymorphisms in genes coding for methylenetetrahydrofolate reductase (MTHFR), adenosine monophosphate deaminase 1 (AMPD1), and several others. [436-440] Interpreting our data is complicated by the fact that the majority of patients (87.3%) were treated with combination therapy a situation that reflects real life practice. There is in fact

current evidence supporting either initial combination or a subsequent step up approach [232,266,441], however the superiority of initial csDMARD combination versus MTX monotherapy remains controversial [442]. In future dissecting key inflammatory/immune pathways at the molecular level are likely to elucidate such heterogeneous responses, including whether differential expression of lymphocytic versus myeloid/fibroblast pathways mediate short and/or long term outcome as previously postulated. [297]

A further element that is worth of note is the significant correlation between the post-treatment reduction in DAS28 and CD68sl. The change in CD68sl is an established biomarker for evaluating response to treatment in established RA [64,332,443]. However this relationship has not been extensively reported in early RA yet, therefore this data is relevant. This suggests that the changes seen in the synovial tissue truly reflect the inflammatory process and the clinical features over time.

Then when analysing US data, I observed a significant correlation between the mean change in DAS28 and the mean change in the PDUS scores, but not between DAS28 and STUS scores. When comparing the mean changes across the three pathotypes, there was a trend toward larger mean fall in PDUS in the Lymphoid group, however this did not reach statistical significance (p=0.08). Anyway there are some important considerations when interpreting this data. Firstly only 74% (47/63) patients had paired STUS and PDUS scores. Secondly a limited set of joints has been examined that could not have reflected with sufficient accuracy the synovial inflammatory process. The incorporation of other joints particularly feet has been advocated by some authors e.g. A. Filer and M.A. D'Agostino to obtain a

realistic picture of the global synovitis (personal communication). However, from a practical perspective, several studies have highlighted the utility of PDUS score of the hand and wrist joints for the monitoring of synovitis, which mostly influence clinicians' decisions in the daily rheumatology practice. [414] Another aspect to be considered is that the sensitivity to change for the US could be lower compared to the clinical composite indices such as DAS28. These findings are in accordance with several studies that, although demonstrating the superiority of STUS and PDUS over clinical examination with regard to reliability in reflecting synovitis, did not show enhanced sensitivity to change for the US parameters compared to clinical parameters. [444,445]

Finally I examined whether baseline synovial pathotype associated with radiographic outcome at 12 months. Again this data should be interpreted cautiously, as only 66% (42/63) patients had paired radiographs available for analysis. Secondly the commencement of biologic therapy in 4 patients within the 12 month follow-up period are likely to have significant impact on the results [257,276]. Future studies integrating an imaging modality like MRI, with the capacity to correlate synovial pathobiology with early markers of joint damage such as cartilage loss, bone marrow oedema and erosions are warranted.

55% of patients in clinical remission at 6 months had persistent PDUS, and this associated with a significantly higher joint erosion score at 12 months. This is in line with previous data demonstrating that persistent synovial neo-vascularity equates to higher levels of radiological damage and reinforces recent initiatives such as US treat to target [227]. A shortfall of this analysis is that I have used an arbitrary definition of US remission (PDUS≤1). The same definition has been

adopted by other groups [222], although most studies have opted for a more stringent one, namely absence of PDUS signal [405,406,446-448]. Indeed the definition of US remission is still vague and remission criteria vary from one group to another, as the minimal accepted level of US remains unknown. [227,376]

In conclusion, this data highlights the potential role of synovial pathotype as a biomarker of response to csDMARD therapy in patients with early RA. This is relevant particularly because none of the laboratory, clinical or imaging findings were predictive of clinical response. Once assessed that the probability of response to early csDMARD treatment is low in specific groups of patients, therapeutic alternatives should be explored.

The present findings support a role for synovial biopsy studies in the stratification of early RA patients. Additional and implemented studies in larger cohorts are needed to replicate these results and provide further insight in the histopathological subtypes of RA and correlations with phenotypic expression.

Chapter 5 : SYNOVIAL PATHOTYPE PREDICTS RESPONSE TO CERTOLIZUMAB PEGOL IN PATIENTS WITH RHEUMATOID ARTHRITIS

5.1 INTRODUCTION

Despite major therapeutic advances over the last two decades, the management of RA remains problematic. Approximately 30–40% of patients do not respond to initial treatment with MTX alone or in combination with steroids and/or other csDMARD. [269] After failure of csDMARD therapy, second line treatment with biologic drugs is commenced, in most cases TNF inhibitors (TNFi). Nonetheless only 70% of patients respond to first line biologic, and only 30% achieve a state of low disease activity or remission [449,450] Moreover, 30-40% of initial responders subsequently develop an inadequate response. [451]

Unfortunately, predictors of response to anti-rheumatic therapy including TNFi are scarce. [350,452,453] Mechanisms of response/non response are largely unknown and treatment strategies are still based on a 'trial and error' approach rather than on prognostic stratification of patients. [382] Given the clinical heterogeneity of RA in addition to variable therapeutic response, factors such as prognostic outcome may need to be incorporated into therapeutic algorithms given the potential toxicity and high cost of biologic drugs. [454]

Currently five TNFi are approved for the treatment of RA in Europe and the US: Infliximab, Etanercept, Adalimumab, Certolizumab pegol and Golimumab. Numerous studies have shown that these agents display similar effects in terms of efficacy and safety profile. [450] However they have distinct pharmacokinetic and pharmacodynamic properties that may affect their biological and clinical properties. Certolizumab pegol (CZP) -the agent utilized in this research project- has a unique biochemical structure, as it is the only pegylated compound among all TNFi. Pegylation of biological proteins, defined as the covalent conjugation of

proteins with polyethylene glycol (PEG), leads to a number of biopharmaceutical improvements, including increased half-life, increased solubility and reduced immunogenicity. [455] In contrast to the other TNFi, CZP does not cause complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC), because of the lack of a Fc region.[456] Animal studies have demonstrated that CZP may also penetrate more effectively into inflamed arthritic tissue than other TNFi. [457]

Anti-TNFα agents interfere with a number of significant pathways driving RA pathogenesis within the synovium: these include local production of chemokines and cytokines [458,459], endothelial expression of adhesion molecules [460,461], regulation of cellular infiltrate [462], production of matrix metalloproteinases [463]. Within this thesis I have demonstrated that both the extent and the pattern of synovial immune infiltration are remarkably variable among different individual with early RA, associate with specific clinical phenotypes and predict response to csDMARD. However whether the synovial pathotypes may be used to stratify patients response to anti-TNFα therapy remains poorly understood. Transcriptome analysis of synovial biopsies prior commencement of treatment showed that different levels of tissue inflammation are associated with different degree of response to TNFα blockade, providing proof of concept that synovial biomarkers can be helpful to discriminate between responders and non-responders. [464] Badot *et al* performed microarray and himmunoistochemistry analysis on 25 arthroscopic synovial tissue from csDMARD-resistant RA patients prior initiation of Adalimumab and evaluated response at 3 months, founding that poor responders were characterised by higher synovial expression of specific

genes involved in the regulation of immune responses. [465] Wijbrandts and colleagues observed that the clinical response to Infliximab was in part dependent on pre-treatment TNFα level in the synovium; the synovial expression of macrophages and T cells, both representing an important source of TNFα, was also significantly higher in responders than in non-responders. [380]

Given the controversial role of synovial lymphocytic aggregates in RA pathogenesis, some studies have specifically focused on the relationship between the presence of such structures and the response to anti-TNFα treatment. Several evidences have indicated that cytokines of the TNF family including LTβ, LTα and TNFα play a key role in the pathways that control the lymphoid tissue development. Pasparakis *et al* reported lack development of splenic primary B cell follicles, follicular dendritic cells and germinal centres in TNFα-deficient mice. [466] In a following report, these observations were extended to other secondary lymphoid organs including Peyer's patches and peripheral lymphonodes, demonstrating the strategic role of TNFα in secondary lymphoid tissue organisation independently of the anatomical or embryological determinants. [467] Although the exact role of TNFα in the organisation of B cell follicles remains poorly characterised, it has been proposed that in TNFα knockout animal models the primary defect may be the failure of follicular dendritic cell precursors to migrate to the follicular area. [468] In addition, Ngo et al have demonstrated that TNFα is required for normal expression of CXCL13 and CCL21, critical chemokines involved in the lymphoid organisation. [469] Klimiuk *et al* have recently reported that synovial tissues containing follicular aggregates show increased transcriptional levels of TNFα compared to diffuse synovitis. [308] They also observed higher

concentrations of TNFα and soluble TNFα receptors in the serum of patients with synovial aggregates, and these levels correlated with markers of disease activity. Further evidence reinforcing these concepts has been recently provided by Thurlings *et al*, who reported a significant increase of TNFα producing cells in the sublining of synovium containing lymphoid aggregates along with increased levels of biomarkers of systemic inflammation. [323]

It has been mentioned that, because of its peculiar structure and pharmacodynamic properties among all the TNFi, CZP may have a different mechanism of action compared to the other agents of the same class. For example, the ability of CZP to penetrate more effectively into inflamed arthritic tissue has been observed on biofluorescence imaging in animal studies. [457] Canete demonstrated the ability of TNFi to revert/disrupt lymphoid structures: of the 16 patients who had a lymphoid pattern in the first biopsy, 9 turned lymphoid negative (5 received Adalimumab, 2 Infliximab and 2 Etanercept) and 7 remained positive at second biopsy after therapy. [330] Whether the superior tissue penetration of CZP could translate into a better ability to penetrate and theoretically disrupt lymphoid structures is unknown and represents an intriguing hypothesis to be explored in further studies.

When looking at the prognostic role of synovial aggregates to predict response to anti-TNFα treatment, the results of current studies are conflicting. Lindberg *et al* analysed 62 arthroscopic biopsies of RA patients prior initiation of Infliximab. They found an overexpression of synovial aggregates (both small and large-sized aggregates) in patients who were good responders according to EULAR criteria at 16 weeks, although this did not reach statistical significance in the prognostic

model. [470] Canete *et al* showed that presence of pre-treatment synovial aggregates was an independent negative predictor of response to treatment in 86 patients with RA (of whom 24 to anti-TNFα therapy). [330] These results are in net contrast with those from Klaasen *et al* who reported that the presence of synovial aggregates was a positive predictor of response to Infliximab in 97 RA patients. [324] However these two studies show profound methodological differences. Firstly, the definition of synovial aggregates was different: Canete considered only highly organised B and T cell aggregates (grade 2 or 3 with specific features of lymphoid structures including T cell/B cell compartmentalization and PNAd positive immunostaining) meanwhile Klaasen included aggregates of any size. As discussed in Chapter 1, this difference is relevant as only B-cell rich large-sized aggregates show the potential to acquire characteristics of ectopic lymphoid structures, meanwhile the biologic significance of small aggregates is uncertain. [322] Moreover, in the Canete's study the cohort was largely heterogeneous including patients at different stages of disease (from early untreated RA to anti-TNFα failure patients, the latter clearly representing a treatment-resistant subgroup) and the outcome was evaluated at different end-points, meanwhile in the Klaasen study all patients were evaluated at a fixed end-point (after 16 weeks of Infliximab therapy) and previous use of TNFα inhibitors was an exclusion criterion.

Herein I would like to assess whether synovial tissue analysis might be helpful to predict the clinical response to anti-TNFα therapy in a cohort of biologic naïve csDMARD-failure RA patients who qualified for anti-TNFα according to NICE guidelines. In the first instance the relationship between the clinical features and the characteristics of the synovium prior commencement of the anti-TNFα agent

CZP will be explored. Then I will investigate whether the presence of a specific synovial pathotype at baseline is a potential predictor of the clinical response at 3 months.

A further aspect that will be explored is the association of synovial features with level of synovitis assessed by US imaging prior and post anti-TNFα therapy. Over the past few years, US and particularly the presence of PDUS signal reflecting synovial vascularization has proved to be a sensitive tool for detecting persistence of active synovitis which is often underestimated by clinical measures. Indeed, it has long been appreciated that joint destruction continues to progress despite the apparent suppression of synovitis as assessed by clinical examination in patients on anti-TNFα. Indeed hypervascularization and overexpression of pro-angiogenetic factors within the synovial tissue are essential pathogenic mechanisms leading to joint inflammation and subsequent damage, and TNFα participates in several mechanisms of neo-angiogenesis and vasodilatation such as local activation of endothelial cells, release of nitric oxide, production of pro-angiogenetic factors and increased vascular permeability. [471-473] Agents that antagonize the effects of this cytokine would be expected to decrease synovial vascularity, reflecting a regression in the inflammatory activity, which is in line with previous findings. [118,474] The potential value of US in monitoring anti-TNFα treatment has been evaluated only in a limited number of studies so far. [388,475-482]

5.2 AIMS AND OBJECTIVES

The hypothesis of this experimental chapter was that in a biologic naive RA cohort synovial pathotype predicts response to the anti-TNFα inhibitor CZP.

The specific aims were to assess whether baseline synovial pathotype significantly associated with the following clinical outcomes after 3 months of treatment:

1) change in DAS28;
2) achievement of EULAR response;
3) change in STUS and PDUS scores.

5.3 MATERIALS AND METHODS

5.3.1 Study population

28 consecutive patients recruited as part of the CLIP-cert study at Barts Health NHS Trust. All patients fulfilled 2010 ACR/EULAR criteria for RA[4] and were eligible for anti-TNFα therapy according to NICE guidelines [http://www.nice.org.uk/nicemedia/pdf/CG79NICEGuideline.pdf]. Inclusion and exclusion criteria for study enrolment are summarised in Table 5.1.

5.3.2 Study design

This was a prospective observational study in RA patients.

INCLUSION CRITERIA	EXCLUSION CRITERIA
- ≥ 18 and years of age	- Women pregnant or breast feeding
- On MTX for at least 4 months, with a stable dose of 7.5-25 mg/week for a minimum of 4 weeks	- Use of any investigational drug within 1 month prior to screening or within 5 half-lives of the investigational agent, whichever longer
- Capable and willing to provide written informed consent	- Serious active infections
	- Active TB or evidence of latent TB without documented adequate prophylactic therapy
	- Presence of a transplanted organ (with the exception of a corneal transplant > 3 months prior to screening)
	- History of malignancy within the past 5 years (except for squamous or basal cell carcinoma of the skin that had been treated with no evidence of recurrence)
	- History of lymphoproliferative disease, or signs and symptoms suggestive of lymphoproliferative disease

Table 5.1: CLIP-Cert study: inclusion/exclusion criteria

5.3.3 Patients assessment

Before study enrolment, all patients were assessed for anti-TNFα eligibility according to NICE criteria and underwent a safety screen prior commencement of therapy as per local guidelines, including: pregnancy test in child-bearing women, Human Immunodeficiency Virus (HIV) screen, HBV and HCV screen, Interferon-Gamma Release Assay (IGRA) test for TB and chest XR.

Patients were assessed before and after 3 months of anti-TNFα therapy.

At baseline demographic data were collected and clinical and laboratory assessment (including RF and ACPA status) completed. US assessment and US-guided synovial biopsy of a swollen joint with relative histopathological analysis were also performed, according to the methodology illustrated in Material and Methods (Chapter 2). A second biopsy of the same joint was performed in 18 patients, and paired histology data were available for 14 patients. As previously described, samples were classified as: 'Lymphoid' based on the presence of large lymphocytic aggregates (G2/G3); 'Myeloid' based on the absence of G2/G3 aggregates and presence of CD68sl (2-4 SQ); 'Pauci-immune' (PI) characterised by absence of G2/G3 aggregates and scarce/absent CD68sl (0-1 SQ).

Standard AP radiographs of the XR of hands and feet were also performed at baseline. The presence of erosions as assessed during routine reporting of radiographs by a musculoskeletal radiologist was also determined.

After 3 months of therapy with CZP, clinical, laboratory and US assessment were re-evaluated; at this time point, clinical and PDUS and STUS outcomes were formally calculated.

5.3.4 Therapeutic protocol

After undergoing synovial biopsy, patients were commenced on CZP subcutaneous injections fortnightly (two induction doses of 400 mg followed by maintenance dose of 200 mg). All patients were on MTX therapy for at least 4 months, with a stable dose of 7.5-25 mg/week for a minimum of 4 weeks, plus Folic Acid ≥ 5 mg/week. Other concomitant csDMARD were permitted. Oral steroids (PRED ≤10 mg/day, or equivalent corticosteroids dose) were allowed if stable for at least one month prior to study enrolment. Use of NSAIDs was allowed during the entire duration of the study.

5.3.5 Outcome measures

Outcome measures were assessed at baseline and following 3 months of CZP therapy.

Primary outcomes were:

1) change in disease activity based on DAS28;
2) achievement of EULAR response.

Secondary outcome was:

- changes in STUS and PDUS scores.

5.3.6 Statistical analysis

Continuous variables were expressed as mean (+/-SD) and ordinal variables as median (+/- IQR). Chi-Square test was used for comparison of qualitative variables between PI, Myeloid and Lymphoid groups and Kruskal Wallis test for comparison of quantitative variables, as appropriate.

Spearman correlation coefficients were used for semi-quantitative variables and Pearson correlation coefficients for continuous variables.

To estimate whether the presence of a specific synovial pathotype at baseline could serve as a predictor of response to TNFα blockade after 3 months of treatment, multivariate logistic regression analysis was performed. The relevant adjusting variables were sequentially selected in the modelling process and the results were expressed as odds ratios (OR) with 95% confidence intervals (95% CI).

The analysis was performed using SPSS (IBM version 21.0 for Mac). All tests were 2-sided and the significance level was set at $p < 0.05$.

5.4 RESULTS

5.4.1 Patient demographics

Baseline demographics and clinical features of patients prior starting anti-TNFα treatment are shown on Table 5.2.

Mean disease duration was 6.2±5.7 years. 53.6% patients were seropositive for RF and 64.3% for ACPA. 42.9% were erosive in either hands or feet.

35.7% were on a stable dose of oral steroids. Patients had been treated unsuccessfully with an average of 1.8±0.8 csDMARD that had been discontinued prior study inclusion due to inefficacy or toxicity.

	N=28 (100%)
Female, n (%)	21 (75%)
Age (years), mean ± SD	51.7 ± 12.3
Onset (years), mean ± SD	6.2 ± 5.7
Smoking, n (%)	12 (42.9%)
ESR (mm/h), mean ± SD	27.1 ± 18.3
CRP (mg/L), mean ± SD	10.4 ± 27.7
RF +, n (%)	15 (53.6%)
ACPA +, n (%)	18 (64.3%)
TJ (28 joints), mean ± SD	16 ± 7
SJ (28 joints), mean ± SD	9 ± 4
DAS28, mean ± SD	6.4 ± 0.8
HAQ, mean± SD	1.57 ± 0.74
Erosive, n (%)	12 (42.9%)
Oral steroid, n (%)	10 (35.7%)
Previous csDMARD, mean ± SD	1.8 ± 0.8

Table 5.2: Demographics and clinical characteristics of CLIP-Cert patients at baseline
Abbreviations: anti-CCP= anti-cyclic citrullinated proteins antibodies; CRP= C-reactive protein; csDMARD= conventional synthetic disease modifying antirheumatic drugs; DAS28= 28 joint count-Disease Activity Score; ESR= erythrocyte sedimentation rate; HAQ= Health Assessment Questionnaire; RF=rheumatoid factor; SJ= swollen joints; TJ= tender joints; VAS-GH= Visual Analogue Score for Global Health.

5.4.2 Baseline synovial pathotype does not define a specific clinical phenotype

The presence of a Lymphoid pattern was found in 11 (39.3%) patients, Myeloid pattern in 4 (14.3%) and PI pattern in 13 (46.4%) (Table 5.3). The proportion of patients displaying large aggregates was in line with other reports cohorts of csDMARD inadequate responder RA patients. [323,324]

I went on to determine whether significant differences in demographics were seen between patients classified according to each synovial pathotype group. I could find no differences in demographic factors including sex, age, disease duration and potentially confounding factor such as smoking. Importantly, no difference with regard to clinical or laboratory features including disease activity, HAQ, erosive status, inflammatory markers and antibody status was detected (Table 5.3). Number of csDMARD previously taken was also similar across the three groups. I did however find that a significantly higher number of patients within the PI group were treated with oral steroids: PI=7 (53.8%), Myeloid=3 (75%) and Lymphoid=0 (0%), $p<0.01$ (Table 5.3).

I also went onto evaluate whether the degree of immune cell infiltration assessed using SQ analysis differed across pathotypes. I found that the presence of a Lymphoid pattern was significantly associated with a higher degree of CD3+ T cells, CD20+ B cells, CD138+plasma cells and CD68+ lining and sublining layer macrophages (Table 5.4). These observations confirm previous data [323], including data from the PEAC study, demonstrating a significant association between a Lymphoid pathotype and higher degree of immune cell infiltration.

	Pauci-immune N=13 (46.4%)	Myeloid N=4 (14.3%)	Lymphoid N=11 (39.3%)	p value
Female, n (%)	10 (76.9%)	3 (75%)	8 (72.7%)	0.97
Age (years), mean ± SD	54.6 ± 10.2	40.5 ± 13.7	52.3 ± 12.7	0.16
Onset (years), mean ± SD	7.2 ± 7.1	8.0 ± 5.7	4.3 ± 3.2	0.52
Smoking, n (%)	6 (46.2%)	2 (50%)	4 (36.4%)	0.84
ESR (mm/h), mean ± SD	27.1 ± 18.9	32.7 ± 26.2	25.1 ± 16.1	0.93
CRP (mg/L), mean ± SD	5.3 ± 4.3	35.0 ± 59.5	7.5 ± 5.6	0.43
RF +, n (%)	7 (53.8%)	2 (50%)	6 (54.5%)	0.98
ACPA +, n (%)	9 (69.2%)	2 (50%)	7 (63.6%)	0.78
TJ (28 joints), mean ± SD	18 ± 7	16 ± 5	14 ± 8	0.45
SJ (28 joints), mean ± SD	9 ± 3	10 ± 3	10 ± 5	0.61
DAS28, mean ± SD	6.5 ± 0.8	6.5 ± 0.7	6.1 ± 0.7	0.40
HAQ, mean ± SD	1.80 ± 0.69	1.12 ± 1.06	1.47 ± 0.63	0.27
Erosive, n (%)	5 (38.5%)	3 (75%)	4 (36.4%)	0.37
Oral steroids, n (%)	7 (53.8%)	3 (75%)	0 (0%)	<0.01
Previous csDMARD, mean ± SD	1.6 ± 0.4	2.2 ± 0.5	1.8 ± 1.1	0.20

Table 5.3: Baseline characteristics of CLIP-Cert patients: comparison across synovial pathotype groups

Values are expressed as mean ± SD or number (percentage). Kruskal Wallis test or Chi-Square test, as appropriate.

Abbreviations: anti-CCP= anti-cyclic citrullinated proteins antibodies; CRP= C-reactive protein; csDMARD= conventional synthetic disease modifying antirheumatic drugs; DAS28= 28 joint count-Disease Activity Score; ESR= erythrocyte sedimentation rate; HAQ= Health Assessment Questionnaire; RF=rheumatoid factor; SJ= swollen joints; TJ= tender joints; VAS-GH= Visual Analogue Score for Global Health.

	Pauci-immune N=13 (46.4%)	Myeloid N=4 (14.3%)	Lymphoid N=11 (39.3%)	p value
CD3	0.5 (0-1)	1.5 (1-2)	3 (3-4)	0.01
CD20	0 (0-0.25)	0.5 (0-1)	3 (3-4)	<0.01
CD68l	1 (0.75-1.25)	1 (0.25-1.75)	3 (3-3)	<0.01
CD68sl	1 (1-1)	2 (2-2)	4 (3-4)	0.02
CD138	0 (0-0.75)	1 (0.25-1)	3 (3-3)	0.02

Table 5.4: Synovial pathotype is associated with degree of immune cell infiltration
Values are expressed as median (interquartile range) of the semi-quantitative (0-4) scores. Kruskal Wallis test.
Abbreviations: CD3, T cells; CD20, B cells; CD68l, lining macrophages; CD68sl, sub-lining macrophages; CD138, plasma cells.

5.4.3 Ultrasonographic power Doppler scores are significantly higher in patients with a Lymphoid pathotype.

Baseline STUS and PDUS scores were available for 20 patients. I could not find any significant correlation between STUS and PDUS scores and clinical and laboratory parameters. However, in line with the findings observed in the PEAC, the relationship between PDUS and level of CD3, CD20, CD68 and CD138 synovial infiltration was relevant. In fact, meanwhile STUS was comparable among the three synovial pathotype groups (p=0.15), PDUS was significantly lower in the PI group (2.5±2.2), compared to the Myeloid (11.6±4.7) and Lymphoid (9.0+6.0) group, p=0.01. Similarly, meanwhile STUS did not correlate with any of the synovial cell subtypes, PDUS significantly correlated with CD3 (r=0.54, p=0.01) and CD20 (r=0.54, p=0.01). Overall these results suggest that PDUS maybe a sensitive measure of pathobiologic diversity.

5.4.4 Baseline Lymphoid pathotype predicts clinical response to Certolizumab-pegol at 3 months

After 3 months of treatment with CZP, clinical response was evaluated by assessment of DAS28 score. Overall the mean fall in DAS28 from baseline to 3 months was -2.3±1.3, with a lower fall in the PI -1.7±1.4 compared to Myeloid (-2.8±1.6) and Lymphoid (-2.8±0.7) group, p=0.03 (Figure 5.1)

I then classified patients as EULAR responders/non responders and found that 20 patients (71.4%) were classified as responders and 8 (28.6%) as non responders. These results are in line with previously reported response rates for other anti-TNFα. [449,450]

I next went onto evaluate whether baseline synovial pathotype predicted EULAR response at 3 months. The results demonstrated that a significantly higher proportion of patients within the lymphoid group at baseline was associated to the chance of achieving better outcome (p=0.01): in particular, the PI pattern was associated with the lowest proportion of patients achieving EULAR response (46%), meanwhile all patients who exhibited a Lymphoid pattern were classified as responders (100%) (Figure 5.2)

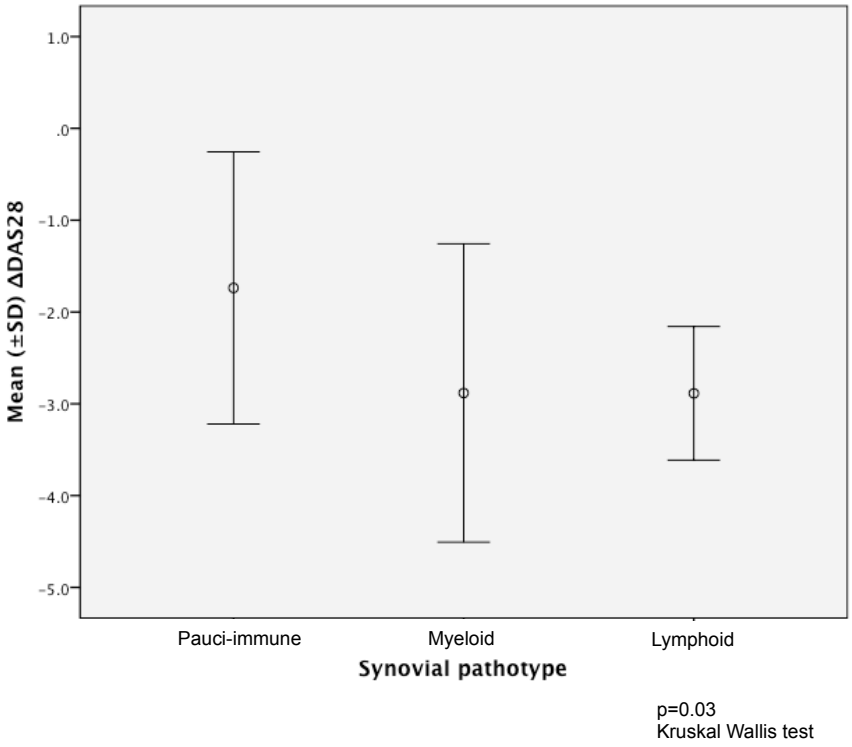


Figure 5.1: Mean fall in DAS28 across the three histopathotype groups
Abbreviations: ΔDAS28= mean change in 28 joint count-Disease Activity Score.

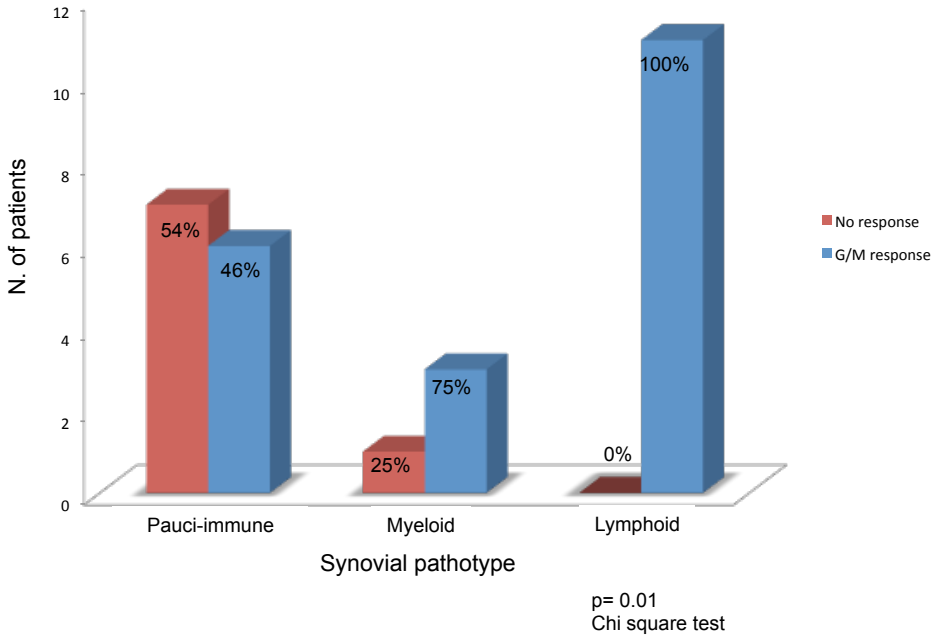


Figure 5.2: Achievement of EULAR response across the three histopathotype groups
Abbreviations: G/M= good/moderate response according to EULAR response criteria.

To assess whether the relationship between the synovial pathotype and the clinical response was independent of other potential factors, a multivariate logistic regression analysis was performed. Relevant study variables entered in the model were: gender, age, disease duration, ESR, CRP, DAS28, HAQ, antibody status, use of oral steroids, erosive status and the synovial pathotype. These variables were firstly individually tested for their association with EULAR response at 3 months using univariate logistic regression analysis. Subsequently multivariate logistic regression analysis was performed using a backward stepwise method. The synovial pathotype was identified as the only potential predictor of response ($p=0.02$). Results are displayed on Table 5.5.

	Univariate model			Multivariate model		
	OR	CI 95%	p value	OR	CI 95%	p value
Gender	0.333	0.033-3.335	0.35			
Age	0.904	0.813-1.006	0.06			
Disease duration	0.948	0.825-1.090	0.45			
ESR	0.981	0.938-1.027	0.41			
CRP	1.068	0.889-1.282	0.48			
DAS28	0.494	0.168-1.450	0.19			
HAQ	0.224	0.046-1.093	0.06			
ACPA+	0.500	0.080-4.000	0.45			
RF+	0.962	0.995-1.005	1.00			
Oral steroids	1.114	0.203-6.105	0.90			
Erosive	0.667	0.128-3.470	0.63			
Synovial pathotype	7.775	1.237-48.859	0.02	9.885	1.352-72.277	0.02

Table 5.5: Association between baseline characteristics and EULAR response at 3 months using univariate logistic regression analysis and multivariate logistic regression analysis

Abbreviations: ACPA= anti-cyclic citrullinated proteins antibodies; CI= confidence interval; CRP= C-reactive protein; DAS28= 28 joint count-Disease Activity Score; ESR= erythrocyte sedimentation rate; HAQ= Health Assessment Questionnaire; OR= odds ratio; RF= rheumatoid factor.

5.4.5 The change in clinical disease activity correlates with the change in the synovial sublining macrophages

In addition to further evaluate whether synovial pathotype associated with treatment response, the correlation between change in DAS28 and change in SQ CD68sl number between baseline and 3 months was determined for all patients with paired histology data (n=14). The results demonstrated a significant correlation between fall in DAS28 and fall in sublining macrophage number (Spearman's r=0.58, p=0.02) (Figure 5.3)

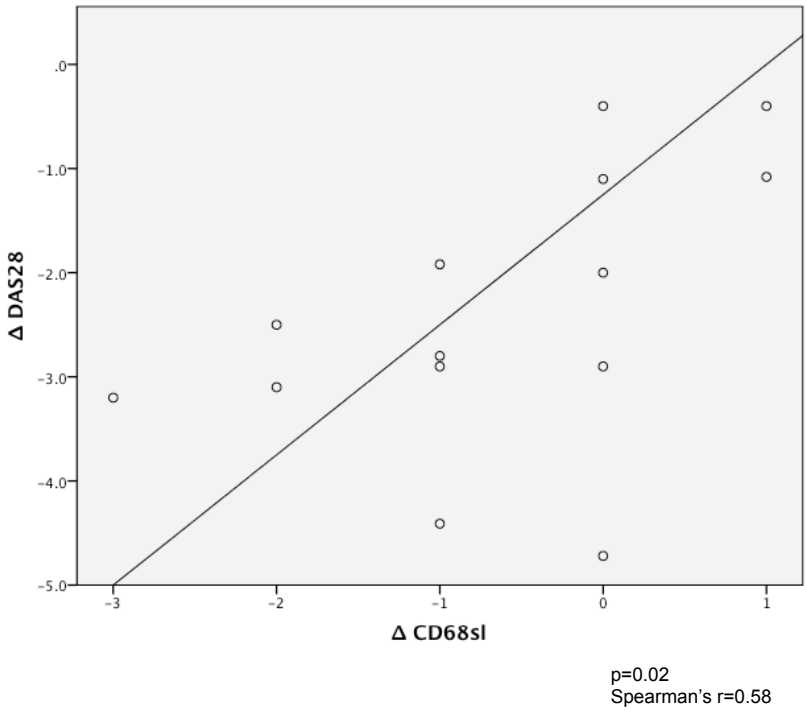


Figure 5.3: Significant correlation between change in DAS28 and change in semi-quantitative sublining macrophages between baseline and 3 months
Abbreviations: ΔCD68sl= mean change in semi-quantitative sublining macrophages; ΔDAS28= mean change in 28 joint count-Disease Activity Score.

5.4.6 A baseline Pauci-immune pathotype is associated with a significantly lower fall in synovial thickening and power Doppler ultrasound scores

Next in order to determine whether STUS and PDUS scores matched changes in disease activity after treatment, change in STUS and PDUS scores were correlated with change in DAS28. In the patients with available paired US data available (n=19) the results demonstrated a significant association with mean change in DAS28 and both mean change in STUS ($r=0.50$, $p=0.02$) and PDUS ($r=0.69$, $p<0.01$) demonstrating that US synovitis scores fell in line with clinical response to CZP. The total percentage of patients in US remission (mean PDUS ≤ 1) was 31.6%.

I then went on to evaluate whether US outcome measures at 3 months were influenced by baseline synovial pathotype. Mean differences in STUS and PDUS between baseline and 3 months follow-up were compared across the three synovial pathotype groups. A significant difference in STUS (mean -2.89±4.78 $p=0.03$) and PDUS change (mean -3.63±5.9, $p=0.04$) across the three groups was demonstrated (Figure 5.4 and Figure 5.5). Of particular interest was the significantly lower mean change observed within the PI pathotype group suggesting a particularly treatment resistant pathotype.

In terms of achievement of US remission I could not observe any significant difference across the three pathotype groups ($p=0.66$).

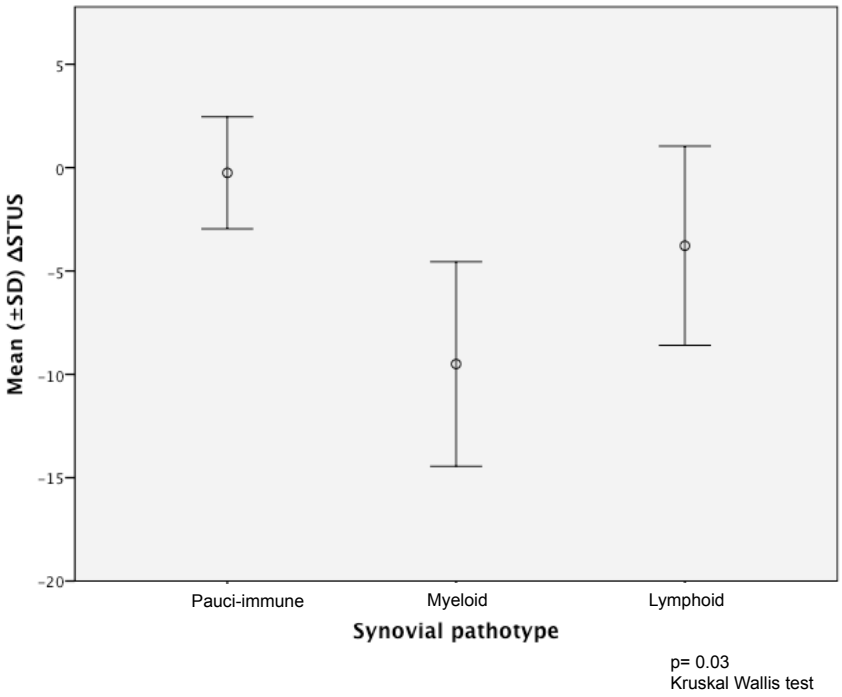


Figure 5.4: Mean fall in STUS in the three histopathotype groups
Abbreviations: ΔSTUS= change in synovial thickening ultrasound.

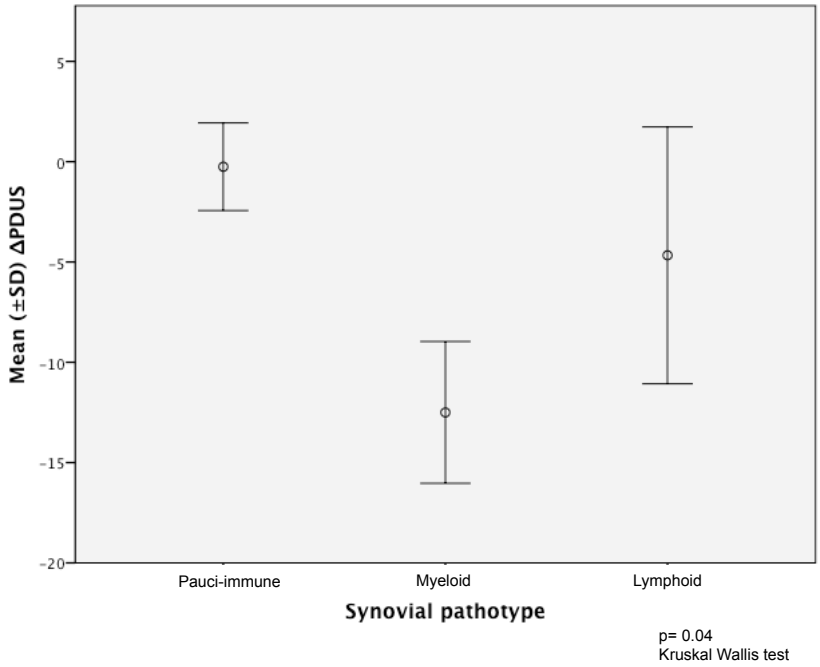


Figure 5.5: Mean fall in PDUS in the three histopathotype groups
Abbreviations: ΔPDUS= change in power doppler ultrasound.

5.5 DISCUSSION

The aim of this work was to investigate the relationship between pre-treatment synovial pathotype and primary response to the anti-TNFα agent CZP in a cohort of csDMARD inadequate responder RA patients.

The results presented herein demonstrate a number of significant findings. Firstly that the proportion of patients with a synovial Lymphoid pathotype within the CLIP-Cert cohort was consistent with that identified within the PEAC. Although the two cohorts are not prospective, this suggests that the formation of lymphocytic aggregates represents an early feature of RA and is not dependent on disease duration.

Secondly, I could not demonstrate any significant association between the presence of a specific histopathotype and indices of disease activity, markers of inflammation and presence of autoantibodies. Similarly to data shown herein, Klaasen *et al* [324] found no association between presence of large lymphocytic aggregates and circulating autoantibodies, serological level of inflammatory markers and clinical features. Thurlings *et al* [323] found that large aggregates were associated with higher levels of ESR and CRP, but not with RF positivity, meanwhile ACPA positivity was surprisingly more frequent in patients with diffuse synovitis. In the cohort described by Canete *et al* [330], patients with aggregates had higher disease duration at baseline, but no association with antibody status, inflammatory markers, disease activity and erosive status was detected. The lack of association with clinical and serological features in the CLIP-cert cohort are in contrast with what observed in PEAC. This may suggest that the immunological functionality and the pathogenetic role of the synovium seen in the early,

treatment naïve phase may be lost after the use of concomitant treatment such as csDMARD and steroids. However, the smaller sample size could be accounted for a low chance to detect statistically significant results.

Thirdly and of particular importance was the observation that synovial pathotype predicted response to CZP at 3 months. This represents a critical decision making point for clinicians as the clinical response to bDMARD is expected to occur within the first 3 months of therapy. Only a minority of patients will show response later, between 3 and 6 months. The response at 3 months has been shown to be a good indicator of achievement of clinical remission at 12 months. Therefore, patients who did not improve or improved only to a minor extent are much less likely to miss treatment target at a later time point and would benefit from a treatment switch. [483] Moreover, the response at later time-points could be more likely affected by confounding mechanisms including the development of human anti-drug antibodies.

Notably when the synovial pathotype was included into a logistic regression model to predict response to therapy, the presence of a Lymphoid pathotype resulted as a strong independent predictor of response. Meanwhile the observation that a PI pathotype associated with a lower chance to achieve response strongly suggests that patients characterised with this pathotype are particularly resistant to treatment with CZP. These observations confirm the original hypothesis that the histopathotype may translate into a specific clinical phenotype with relevant prognostic implications.

Furthermore, as previously reported in various studies [64,332,443], this data confirms a significant correlation between post- treatment changes in DAS28 and

changes in CD68sl, suggesting that synovial macrophages represent a candidate biomarker to predict efficacy of anti-rheumatic treatment.

Of note, because CZP is the only pegylated drug among TNFα inhibitors and pegylation confers specific pharmacological properties, including more effective distribution and retention within the inflamed joint tissue, it cannot be inferred that the results obtained herein could be extended to other anti-TNFα, and further studies are necessary in that respect. A study conceptually and methodologically similar to CLIP-cert but based on the use of an alternative TNFi (Enbrel) is currently ongoing in our department. It will be important to compare the findings of these two works, however further and larger studies would be required.

When looking at the association between the synovial pathotype at baseline and the change in STUS and PDUS after therapy, I observed that PI was a particularly resistant pattern to change. Again this group appears to have a pathological status whereby the control of mechanisms that drives synovitis has not been achieved by therapy, and, on the opposite, that the other two groups are more responsive to the immunomodulatory effect of anti-TNFα treatment. However, data should be evaluated with caution as the baseline levels of STUS and PDUS in the PI group were lower compared to the other 2 groups ($p=0.15$ and $p=0.01$, respectively) and this could explain a less sensitivity to detect changes in this specific group. Also, the small number of patients within this study is likely to be underpowered to detect a real difference. Therefore these findings are only exploratory and require further confirmation.

The final observations reported regarding clinical, pathological and US correlates are also of significance. Firstly the observation that changes in DAS28

and PDUS and STUS correlations were significantly associated provides an objective measure of disease outcome in this observational study. Previous studies reported that synovial tissue tends to become chronically thickened and less reversible in long-standing RA. [406] There are few reports of sustained regression of ultrasonographic synovial thickening after TNFα blockade. For example, in a pilot study assessing 11 patients with active RA prior and after six weeks of Infliximab therapy, a significant decrease in both STUS and PDUS was observed (wrists, MCPs and PIPs). [477] In line with these data, the results I have shown herein demonstrate that chronic synovitis measured by STUS is capable of being modulated by effective treatment.

Although the expression of angiogenic factors was not evaluated within this study, previous observation that ultrasonographic features of synovitis associate significantly with the expression of factors such as VEGF, Angiopoietin 2 and Tie-2 is of relevance.[404] These factors are critical mediators of angiogenic development/maintenance. As synovial lymphocytic aggregates have been noted to be a significant source of angiogenic promoters [316] this maybe the mechanism by which the significantly higher baseline levels of STUS and PDUS are observed within the Lymphoid group.

A particular strength of this work is the homogeneous cohort of RA patients with participants presenting similar clinical indices including disease duration, number of previous csDMARD, concomitant MTX and clinical features including presence of autoantibodies and level of disease activity. Also, we have been using a novel biopsy technique for high quality synovial tissue extraction, which ensured

a fairly representative cohort of small joints and large joints, and a validated scoring system for histopathological analysis and US data analysis.

This work has some limitations though. Firstly, the observational nature of the study. Secondly, findings are preliminary because of the small sample size. The clinical relevance of this data has to be proven in further larger studies. The present study was aiming to represent the platform for larger and more detailed works currently ongoing in our department, including a new study called STRAP (Stratification of Biologic Therapies for RA by Pathobiology) where csDMARD failure patients are stratified to Etanercept, Rituximab and Tocilizumab aiming to identify synovial predictors of response/resistance to different first line biologic strategies (http://www.matura-mrc.whri.qmul.ac.uk/). Thirdly, I have used a limited joint set for US evaluation that could not be faithfully representative of the disease in its entirety. However there is still lack of knowledge in the scientific community on how to best use US imaging in the context of clinical and research studies, including the minimum joint set to be examined. Our joint set has been used in previous studies so it is not without validation. [370]

In conclusion, these findings highlight the pathophysiological and clinical significance of synovial pathotype as a potential biomarker of response to CZP in patients with RA. Future work should expand the search for other biomarkers and molecular networks, including genetics, epigenetics and proteomics analysis on a larger scale of patients and using randomised control trials. To assess changes within the synovial membrane over time, it would be critical to extend the histopathological analysis of the post-treatment biopsy. In particular, it would be crucial to see if lymphocytic aggregates may be reversed by effective therapy and

if such reversal is a good marker of persistence of the clinical response over time. The post-treatment biopsy may also represent an ideal platform to investigate synovial biomarkers of response/resistance in patients who are due to commence a second line biologic after the failure of the first line. The ultimate aim is to provide a tailored approach to treatment decisions at each stage of the disease, in order to maximise the potential response to therapy and provide the right drug to the right patient at the right time.

Chapter 6 : GENERAL DISCUSSION

Considerable patient-to-patient variation exists at the clinical and biological level in RA. However whether the underlying tissue differences could impact the clinical phenotype and define prognostic categories is not known. The main focus of this thesis was to evaluate how the heterogeneity of synovial pathotypes integrates with the heterogeneity of clinical phenotypes including the heterogeneous response to treatment.

Personalised medicine based on targeted strategies for the individual patient is one of the goals set for future healthcare. Current prognostic markers for RA are unable to reliably predict those patients who would be most likely to respond to individual therapeutic interventions, so additional prognostic markers are needed in order to select the most effective therapy for that specific patient avoiding treatments that could be ineffective or harmful. Since synovial tissue is the epicentre of arthritis pathology, a deeper understanding of the pathobiological processes that initiate and perpetuate joint inflammation is crucial to achieve this goal in RA. The pathobiological processes may differ between patient subgroups or even between different stages of the disease in the single patient. There is in fact accumulating evidence that RA behaves differently in different phases of the disease, and particularly that early RA represents a clinically and biologically distinct phase.

I have examined a cohort of 63 early RA (PEAC) patients who were naïve to treatment prior starting csDMARD, and a cohort of 28 patients with established disease (CLIP-Cert) who failed csDMARD and were eligible to start anti-TNFα. The first major component of this thesis was to examine whether the presence of a specific synovial pathotype at baseline correlated with a specific clinical phenotype

and was able to predict response to csDMARD treatment at 6 months in the PEAC and to anti-TNFα at 3 months in the CLIP-Cert. I am able to report a number of observations:

- Firstly that RA exhibits similar histopathological findings in both the early and long-standing phase, with three histological subsets identified: Lymphoid, characterised by the presence of large lymphocytic aggregates; Myeloid, characterised by the absence of aggregates but significant expression of sub-lining macrophages (CD68sl); and Pauci-immune (PI), characterised by scarce/absent lymphocytic and macrophage infiltration.
- Secondly that the presence of a specific synovial pattern correlates with markers of systemic inflammation and expression of autoantibodies in the PEAC but not in the CLIP-Cert.
- Thirdly that the presence of a Lymphoid pattern is a robust prognostic marker of response to treatment in both the PEAC and CLIP-Cert, meanwhile the Pauci-immune group represents a particularly resistant to treatment subset.
- Fourthly, the change in post-treatment synovial CD68sl correlates with the change in clinical disease activity in both cohorts.
- Finally, the synovial pathotype correlates with ultrasonographic expression of active synovitis at baseline, confirming that US is a faithful measure of synovial pathology. Moreover, change in US measures of synovitis after therapy is lower/ virtually absent in the Pauci-immune group compared to the Lymphoid and Myeloid group confirming that this pathotype represents a resistant subset.

There is pre-existing evidence suggesting that histological classification of synovial tissue ranging from a diffuse to aggregate infiltrate associates with clinical phenotype. However several studies so far have led to conflicting or inconclusive results. [324,330] Interpretation of such data is complicated though by a number of inherent methodological discrepancies related to the uncontrolled nature of the studies and the heterogeneity of the cohorts. Most of these works were conducted on patients at end-stage disease and previous exposure to heterogeneous therapies, with a significant number of biases introduced. Few findings have been validated with subsequent cohorts in early treatment naïve patients. I have analysed a cohort of early RA patients naïve to treatment and, as comparison, a non longitudinal cohort of long standing RA patients which were similar for clinical characteristics, disease duration and exposure to previous csDMARD. A minimally invasive US-guided biopsy technique has been utilised to extract synovial tissue from inflamed joints. This technique confirmed a reliable method of acquiring synovial tissue from both large and small joints [287] proving to be safe and well tolerated by patients with yield of good quantitative and qualitative synovial tissue. US can also facilitate the selection of sampling sites in order to allow extraction of sufficient quantity and good quality tissue. Specifically, the presence of higher synovial thickening scores is associated with better tissue yield. We have followed this rule empirically in the present study, however this has been formally demonstrated in our further work recently. [300] The use of US-guided synovial biopsy has now been validated in several cohorts, but the gold standard for joint tissue extraction remains synovial arthroscopy. [289] It will be necessary to carry out head-to-head studies comparing US-guided biopsy with the

standard technique before adoption on large scale is recommended. Such studies are planned as part of the EULAR synovitis study group and OMERACT research agenda.

The tissue sampling and the histology analysis method utilized herein were rigorous. The histological classification adopted is arbitrary however based on recent new learning on synovial tissue pathology, to which our group has contributed greatly. [297] Historically studies based on synovial tissue analysis have focused on the classic paradigm of mutual distinction between two pathotypes: diffuse (inflammatory cells randomly distributed within the synovium) and aggregate (lymphocytes clustering in discrete entities resembling ectopic lymphoid structures). We have observed that the diffuse group -intrinsically characterised by the absence of lymphocytic aggregates- could be split up in at least two further groups according to the presence/absence of macrophages in the sublining. This supports the concept that synovial heterogeneity exists as a continuous spectrum rather than discrete subsets of biological processes. I have therefore presented a novel histological classification of the RA synovium, based on the presence/absence of lymphocytic aggregates and further on the presence/absence of sublining macrophages (CD68sl) resulting in a more precise evaluation of the synovial pathology compared to the classic distinction in aggregate/diffuse synovium. Indeed there is accumulating evidence of a key role of macrophages in RA. These cells play a pivotal role in the innate response as well as in the adaptive response, by acting as effective antigen presenting cells. They are key in triggering and perpetuating the NF-kB response and related cytokines network including anti-TNFα. Several studies have observed that baseline levels of

synovial macrophages and TNFα gene expression are correlated with clinical outcome. [324,380] Further studies have demonstrated that the change in the number of synovial CD68sl is a potential biomarker to predict response. [64] However this relationship has not been extensively reported in early RA cohorts as yet. The results presented herein show that changes in the number of synovial sublining macrophages correlate with clinical improvement both in the PEAC and the CLIP-Cert, so it appears independent of therapeutic exposure and disease stage.

The frequency of the three synovial pathotypes described is similar in the PEAC and CLIP-Cert. Therefore I provide evidence that synovial aggregates are present during the early phase of the disease and can persist after csDMARD treatment. However, despite several studies including our own work using the SCID mouse model[325] having suggested a role for synovial aggregates in the promotion of autoimmunity at the tissue level, the functionality of these structures is debated and their role in the initiation and perpetuation of the disease is not entirely defined. Findings from the PEAC support a direct pathogenetic role, since a significant association between the synovial pathotype and biomarkers of systemic inflammation and autoantibodies has been observed. Conversely, I could find no association between histopathology and biochemical features in the CLIP-Cert. These discrepancies may be explained by disease stage differences supporting the notion that synovial aggregates might play different roles in early versus late disease. This may reinforce the concept that indeed early RA represents a distinct phase. However addressing the correlations between histological features and clinical and biochemical markers on a historical cohort of patients with varying disease duration and on a number of previous csDMARD is difficult.

Notably though despite a lack of association with inflammatory markers and autoantibodies at baseline in the CLIP-Cert, the histological features do not remain uncoupled from clinical behaviour. Indeed in both the PEAC and the CLIP-Cert the synovial pathotype is significantly associated with change in disease activity expressed by DAS28. Importantly, when several variables including clinical, serological, radiological and ultrasonographic measures are put into a multivariate logistic prognostic model, synovial pathotype represents the strongest predictor of clinical response in both cohorts. Specifically, the presence of a Lymphoid pattern at baseline is associated with the highest chance of achieving a primary response to both csDMARD and anti-TNFα. Dennis et al recently reported a classification of the RA synovium based on four synovial categories designated as lymphoid (B cell dominated and associated with presence of lymphocytic aggregates), myeloid (macrophage and NF-κ B process dominated) low inflammation (intermediate characteristics of the previous two subsets) and fibroid (comprising hyperplastic but scarcely infiltrated tissue), observing that the myeloid but not the lymphoid group was significantly associated with good clinical response to anti-TNFα therapy. [312] At a first glance this seems conflicting with our results, however the above classification was based on gene signature expression, which is profoundly different from a purely histological classification. Particularly the definition of lymphoid and myeloid in the above mentioned study was derived by gene expression profiles marking clear-cut distinction between the lymphoid and myeloid immune axis. The classification that I have adopted herein is not based on gene signature expression. The difference between Lymphoid and Myeloid pattern, for example, is based on the presence/absence of large lymphocytic

aggregates rather than on the expression of cells/pathways derived from the lymphoid or myeloid lineage. Macrophages are abundantly expressed in the Lymphoid subset as well leading to a significantly overlap in terms of myeloid axis/pathway expression across the two groups. Ideally histological analysis should be implemented with more sophisticated analysis including genomics and proteomics. The present study is aiming at representing the platform for further detailed analysis that is currently ongoing in our department.

From the present work it emerges clearly that the presence of a Pauci-immune pattern is associated with a lower chance of responding to treatment. It indeed makes sense that patients with lower levels of synovial infiltrate may present with lower degree of response to both csDMARD and anti-TNFα therapy. This suggests that other factors contribute to determining synovitis namely activation of alternative immunological pathways and release of different inflammatory mediators. A number of other biologic agents are currently available other than TNFα. Thus, it is conceivable that this subset of patients could responder better to alternative treatment e.g. targeting the IL-6 or IL-17 axis which are activated at the stromal level, or even novel pathways that synovial tissue analysis may contribute to the discovery of. Further work is necessary to explore the main inflammatory pathways driven in this group of patients other than, for example, the canonical NF-κB signalling which is predominantly targeted by csDMARD and anti-TNFα.

However, the prediction value of the synovial pathotype presents some limitations. For example, the synovial pathotype did not predict damage progression over a 12 months period in the PEAC. A significant association was previously reported in established RA between synovial aggregates and erosive

burden[329] but the same authors and others in larger cross sectional cohorts found no association between synovial aggregates and radiological damage. [323] We recently reported an association between the presence of synovial aggregates and the erosive burden in a larger early RA cohort that was also part of the PEAC platform. [381] Potentially, a larger sample size and an extended follow up period could have led to similar conclusions herein.

Finally, the PDUS scores were associated with the overall pathology in the synovium. PDUS was highly significantly related to the degree of change in clinical disease activity after treatment in both the PEAC and CLIP-Cert cohorts, as previously reported by other authors. STUS scores changed in parallel with disease activity in the CLIP-Cert cohort only. One could have expected to observe this correlation rather in the PEAC, where collagenous thickness, fibrosis and other changes typical of long-standing disease are less pronounced. A number of reports have indeed shown a disparity of US remission rate in early versus long standing disease, as synovial tissue become chronically thickened and less reversible with disease progression. [406] However, synovial thickening is also the result of neoangiogenesis and cellular infiltration that can revert dramatically after effective anti-TNFα therapy. These results should be interpreted cautiously, primarily because of the limited sample size. Secondly, although US has been performed by expert ultrasonographers and the process of imaging and scoring acquisition was rigorous, these results are based on arbitrarily selected number of joints (bilateral wrist and MCP joints) which lacks validation. The performance of the limited joint set has not been compared with an extended joint set, which should have included for example feet joints, as advocated by experts in this field.

[372] Correlating the histology and the US data at the single joint level could have also added further relevant information, but this aspect has not been included in the present analysis.

In the work I have presented, synovial pathobiology added significant prognostic information to clinical, serological and imaging parameters, resulting as the strongest prognostic factor of response to treatment at 6 months in the PEAC and at 3 months in the CLIP-Cert, which represent crucial time-points to make clinical decision including switching to alternative treatment. Predicting which patients are likely to respond according to the pre-treatment synovial pathotype would be of invaluable utility. It seems realistic to suggest that in future synovial pathobiology will be integrated into current clinical prediction models with great benefits for patient care and health economics. The next step would be the integration within more sophisticated clinical prognostic model incorporating more detailed molecular and genetic data and validation in large patient cohorts. Especially further examination on large scale prospective studies of early arthritis, treatment naive patients are needed. Also, I have reported a single-centre experience, and further work is needed to confirm these findings across multiple centres.

In conclusion, the present thesis provides evidence to support that synovial pathobiology could represent the key to personalised healthcare in RA thus suggesting potential application in future studies. At the present time, stratification of RA patients in clinical trials is based on parameters (disease activity, autoantibodies, radiographic evidence of erosions, acute phase reactants) that are associated with disease severity, yet are poorly discriminant on an

individual basis. This data provides a strong rationale for including synovial analysis in the stratification of patients according to prognostic and therapeutic response categories, in view of the ability to identify patients with a higher/lower chance to respond to treatment. Histological assessment has become part of standard clinical care in several chronic autoimmune diseases (e.g. lupus nephritis and vasculitis) or cancer medicine, with management decisions resulting from the integration of pathobiology into clinical, biochemical and imaging features. In a similar way, incorporating synovial analysis in the decision making strategy may revolutionise the management of RA patients in the near future optimising the delivery of personalised care.

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